The Control of Varicella-Zoster Virus Immediate Early Gene Expression.

BY

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SUMMARY

Analysis of the *cis*-acting motifs and *trans*-acting factors responsible for regulating the expression of the varicella-zoster virus (VZV) major immediate early (IE) gene was undertaken. The control of VZV IE gene expression has relevance to the fields of eukaryotic gene regulation and herpesvirus biology.

In order to analyse the control sequences of the VZV major IE gene, knowledge of the location of the 5' end of its mRNA was required. Primer extension and S1 nuclease analyses concurred in defining it as being 74 base pairs (bp) upstream of the proposed translation initiation site. Analysis of DNA sequences upstream of the start site revealed a candidate for a 'TATA' motif present at -25 to -30 bp, a finding common to many genes transcribed by eukaryotic RNA polymerase II.

Analysis of the activity of the control region of the VZV major IE gene was undertaken using plasmids in which sequences to -1150 were inserted upstream of the "reporter" genes encoding β-galactosidase or chloramphenicol acetyl transferase. The activities of the complete control region and of a series of deletions were then determined in a range of cell types, using short term transfection assays. A significant finding to emerge from these experiments was that the control sequences responsible for the transcription of the VZV major IE gene do not direct a high level of expression of reporter genes in any of the cell types tested. The baseline level of transcriptional activity was dependent on sequences located between nucleotides -131 and -25. Co-transfection of a plasmid which expresses the herpes simplex virus type 1 (HSV-1) protein Vmw65 resulted in a 20-50 fold stimulation of expression from the VZV IE promoter. This effect depended on the presence of regulatory sequences between -409 bp and -130 bp. A second set of co-transfection experiments investigated the effect on the VZV control sequences of a plasmid encoding the transactivating product of the adenovirus 5 E1A gene. The presence of this polypeptide resulted in stimulation of expression by 10 fold. In contrast with the effect of

Vmw65, the activity of the E1A gene product depended on sequences present within the first 131 bp of the mRNA start site.

Gel retardation and DNAase I protection assays were used to locate sites at which proteins bind to the control sequences. Results from these experiments reinforced and expanded the conclusions made from the functional assays. DNA fragments from the promoter region, -35 to -130, were shown to bind two proteins. Sequence analysis and competition experiments identified these proteins as belonging to two families of transcription factors known as 'CCAAT box binding' proteins and the 'ATF/CRE' family.

A second protein binding region was present between -409 and -246 and was shown to consist of two binding sites for the cellular transcription factor Oct-1, which binds to the consensus ATGCAAAT. The proximal binding site could be expanded to incorporate a TAATGARAT motif known to allow Vmw65 to participate in the formation of a complex including Oct-1 and other cellular proteins, thus allowing Vmw65 mediated transactivation. Both sites were shown to bind Oct-1 and the proximal site was also shown to bind Vmw65 with high affinity, probably explaining the effect of this protein on VZV IE gene expression.

In view of these results the analysis of VZV open reading frame (orf) 10 was undertaken. Sequence comparison of orf10 and Vmw65 had shown considerable homologies between the amino-terminal portions of the proteins but the absence from orf10 of a carboxy-terminal region of 78 amino acids known to be essential for transcriptional activation by Vmw65. Both the functional activity of orf10 and its ability to participate in complex formation at TAATGARAT motifs were tested. A plasmid, constructed to express the product of VZV orf10, failed to stimulate expression from plasmids containing HSV-1 or VZV IE control sequences. The product of VZV orf10 was also produced <u>in vitro</u> and failed to participate in the formation of DNA binding complexes when incubated with cellular proteins and the TAATGARAT motif.

Two other VZV orfs were studied. The first was number 66, which encodes a protein which shows many similarities to known protein kinases. The system chosen to express this

orf utilised the HSV-1 temperature sensitive mutant tsK and HSV-1 IE control sequences. At nonpermissive temperatures tsK overproduces IE polypeptides, generating quantities sufficient for biochemical characterisation of heterologous orfs expressed in this way. In order to simplify the selection of recombinant viruses containing VZV orfs the E. coli B-galactosidase enzyme, with HSV-1 IE control sequences, was inserted into the thymidine kinase gene of tsK. This done, orf66 was recombined into the virus and progeny selected by their inability to metabolise the chromogenic substance X-gal. Virus stocks containing the VZV orf were purified and its presence confirmed by Southern blot hybridisation analysis. No product of the inserted orf was detected when viral proteins, synthesised at 38·5°C, were analysed by SDS-polyacrylamide gel electrophoresis. The second orf investigated was number 36, which is predicted to encode the VZV pyrimidine deoxynucleoside kinase enzyme. Attempts were made to overexpress and analyse this enzyme using the plasmid pKK233-2, which allows inducible expression of unfused proteins in prokaryotic cells. High levels of expression were achieved but no novel kinase activity could be detected. The possible reasons for this finding are discussed.

ABBREVIATIONS

A	adenine
aa	amino acid
AP1	activating protein 1
APS	ammonium persulphate
ATF	activating transcription factor
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
BZLF	BamHI z left frame
C	cytosine
cAMP	cyclic adenosine-5'-monophosphate
CAT	chloramphenicol acetyl transferase
CBP	CCAAT binding protein
C/EBP	CCAAT/enhancer binding protein
cpe	cytopathic effect
CRE	cAMP response element
CTF	CCAAT transcription factor
Cys	cysteine
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DATD	N,N'-diallytartardiamide
dl	deletion
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dPyK	deoxypyrimidine kinase
DTT	dithiothreitol
E	early
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	sodium ethylenediamine tetra-acetic acid
EEB	electroelution buffer
EHV	equine herpes virus
g	gravity
G	guanine

GB	gel buffer
gD	glycoprotein D
GEB	gel elution buffer
H2b	histone 2b
HCMV	human cytomegalovirus
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane
HFL	human foetal lung
His	histidine
HSV	herpes simplex virus
HTH	helix-turn-helix
IE	immediate early
in	insertion
kb,kbp	kilobase(s), kilobase pair(s)
L	late
L	long
MCMV	murine cytomegalovirus
min	minute(s)
mRNA	messenger ribonucleic acid
NF	nuclear factor
NP40	nonidet p40
OBP	octamer binding protein
OD	optical density
orf	open reading frame
ori	origin of replication
OTF	octamer transcription factor
PAGE	polyacrlyamide gel electrophoresis
PBS	phosphate buffered saline
PIPES	piperazine-N,N'-bis(2-ethane sulphonic acid)
poly dI	polydeoxyinosine
poly dC	polydeoxycytosine
R	purine
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	ribosomal ribonucleic acid
S	short
SDS	sodium dodecyl sulphate
SGB	stacking gel buffer
snRNA	small nuclear ribonucleic acid
SV40	simian virus 40
T	thymidine

IEC - Immediate early complex

NBRF - National Biological Research Foundation

TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylethylene diamine
TFIIIA	transcription factor III A
TK	thymidine kinase
TPA	12-O-tetradecanoyl-phorbol-13-acetate
Tris	tris(hydroxymethyl)aminoethane
ts	temperature sensitive
TS	thymidylate synthetase
U	unique
UAS	upstream activating sequence
UV	ultraviolet
Vmw	molecular weight of viral polypeptide in kilodaltons
VZV	varicella zoster virus
v/v	volume/volume
wt	wild type
w/v	weight/volume

1. INTRODUCTION

1.1. The Human Herpesviruses.

1.1.1. Herpesviruses - Description and Classification.

Herpesviridae are a group of large, DNA containing, viruses which possess a similar overall structure consisting of envelope, tegument, capsid and core (DNA-protein complex)(Wildy et al., 1960, Epstein 1962).

The envelope is a large, loosely fitting, structure derived from the nuclear membrane of the infected cell. It is typically triple layered in section and has projections 8nm in length (Morgan et al., 1959, Epstein, 1962, Wildy and Watson 1963, Asher et al., 1969, Spear and Roizman, 1972). The structure found between the envelope and the capsid is known as the tegument. Its structure on electron microscopy is ill defined, though a fibrous appearance is often observed on negatively stained material (Wildy et al., 1960, Epstein, 1962, Roizman and Furlong, 1974). The nucleocapsid lies within the tegument and has been studied in most detail in HSV-1. It is an icosahedral structure, 100-110nm in diameter, consisting of an outer layer of 150 hexameric and 12 pentameric capsomeres (Wildy et al., 1960) and an inner layer of proteinaceous material in a T=4 icosahedral lattice (Schrag et al., 1989). The herpes virus core is located within the capsid and contains the DNA and protein arranged in a toroidal structure (Epstein, 1962, Nazerian, 1974, Furlong et al., 1972). The most widely accepted model for the structure of this component is that of Furlong et al., (1972) who described a centrally located cylinder, likely to be proteinaceous in nature, around which is wound DNA probably, in the form of a nucleoprotein filament.

The classification of herpes viruses was made on the basis of their biological properties including host range, length of reproductive cycle, cytopathology and characteristics of latent infection, by the Herpes study group appointed by the International committee on taxonomy of viruses (Roizman et al., 1981). Table 1. shows a summary of the characteristics of herpesvirus subfamilies together with the human herpes viruses which belong to them.

Table 1.1.

CHARACTERISTICS OF HERPESVIRUS SUBFAMILIES AND DESIGNATION OF HUMAN HERPESVIRUSES

Alphaherpesvirinae

Short reproductive cycle

Wide or narrow host range in tissue culture

Cytolytic infections

Latency frequently established, primarily in ganglia

Herpes simplex virus 1

Herpes simplex virus 2

Varicella-zoster Virus

Betaherpesvirinae

Relatively short reproductive cycle; slowly progressing infection in tissue culture

Narrow host range in tissue culture; frequently restricted to natural host

Cytomegalic; infected cells undergo enlargement

Human cytomegalovirus

Human herpes virus 6

Gammaherpesvirinae

Host range limited to family or order of natural host

In tissue culture replication in lymphoblastoid cells; some cause lytic infections in epithelioid and fibroblastic cells

Latent infections, often in lymphoid tissues

Epstein-Barr Virus

1.1.2. Human Herpesviruses and Disease.

The six human herpes viruses thus far recognised cause a variety of conditions ranging from the mild to the fatal. HSV-1 and HSV-2 normally cause a subclinical primary infection of mucocutaneous membranes or traumatised skin. A latent infection is then established in the sensory ganglia serving the area of primary infection, with recurrent infections also occurring in this distribution (Baringer and Swoveland, 1973, Fraser et al., 1981). HSV-1 normally affects the oropharynx or the eyes, while HSV-2 infections are mainly localised to the mucous membranes and skin of the genitals (Whitley, 1985).

Varicella-zoster virus (VZV) is the cause of the childhood condition of chickenpox, a widespread vesicular rash present mainly on the head and trunk, though mucous membranes can also be affected. It is spread either by direct contact or as an airborne infection (Johnson et al., 1982). Again, latency in sensory ganglia follows the primary infection and reactivation can lead to the painful disease of shingles (Hope-Simpson, 1965). This can occur at any time following primary infection though it does so more commonly with advancing age, a finding that may be related to decreased immune defence (Schimpff et al., 1972). Both herpes simplex viruses and VZV can spread to the central nervous system (CNS) and cause meningitis and encephalitis, potentially life threatening conditions (Kennedy, 1987).

Human cytomegalovirus (HCMV) infection is normally a benign, self limiting or asymptomatic infection. However, transplacental transfer of HCMV, which can occur as a result of both primary infections and reactivation of latent virus, is, medically, the most important congenital viral infection with intrauterine death, prematurity, malformations and somatic and mental retardation being potential sequelae (Alford and Britt, 1984). Reactivation of latent virus can occur as a result of immunostimulation or immunosuppression and, like primary infections, results in a range of conditions including simple pyrexia, infectious mononucleosis and hepatitis (Ho, 1982).

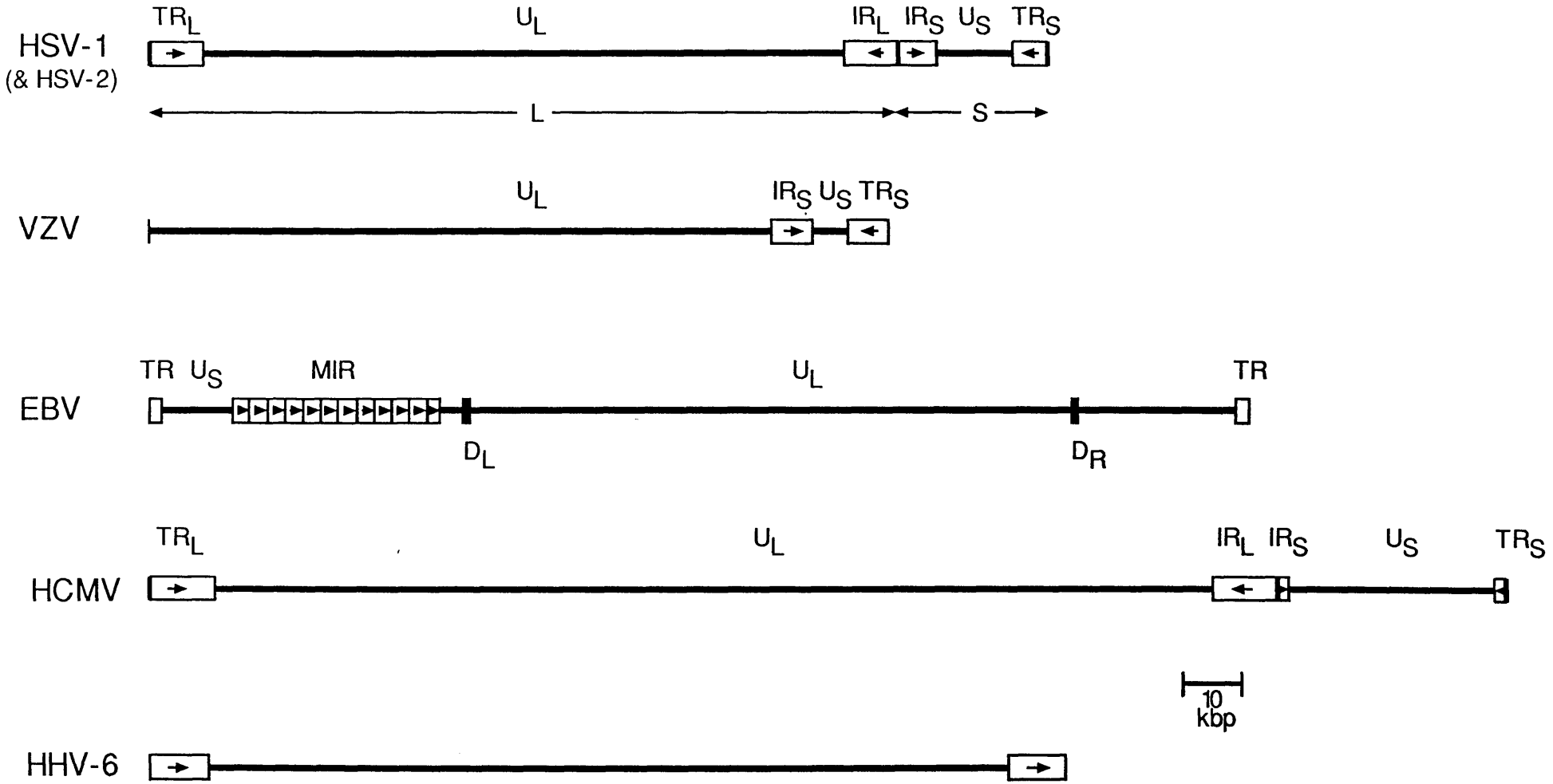
Figure 1.1. STRUCTURES OF THE GENOMES OF THE HUMAN HERPESVIRUSES

The genomes are represented as linear molecules. The bold lines represent unique sequences and the boxes represent repeat sequences with arrows indicating the relative sequence orientations. The genomes are described in Section 1.1.3..

ABREVIATIONS:

Sequences; U_L - unique, long
U_S - unique, short
I/TR_L - internal/terminal repeat long
I/TR_S - internal/terminal repeat short
D_L - direct repeat, left
D_R - direct repeat, right
ori -

This figure was adapted from McGeoch (1989), with permission.


HSV-1
(& HSV-2)
TRL
UL
IRL
IRS
US
TRS
L
S
VZV
UL
IRS
US
TRS
EBV
TR
US
MIR
UL
TR
DL
DR
HCMV
TRL
UL
IRL
IRS
US
TRS
10
kbp
HHV-6

Human herpes virus 6 has been isolated from patients with lymphoproliferative disorders and can cause the condition exanthem subitum, presumably as a manifestation of primary infection (Yamanishi et al., 1988).

Epstein-Barr virus (EBV) usually causes subclinical primary infections, though increasing age is generally associated with increasing severity of symptoms resulting in infectious mononucleosis amongst adolescents. This virus has also been found to be associated with the development of two tumours, Burkitts lymphoma and nasopharyngeal carcinoma, though the precise role played by the virus in these conditions is not clear (Neideman et al., 1976; Miller, 1985).

1.1.3. Genome Structures of the Human Herpes Viruses

The genomes of all herpes viruses are linear DNA molecules which differ amongst themselves with respect to size, base composition, and the pattern of large scale repeated sequences (Honess, 1984). No unique value for genome size can be given as all herpes viruses so far sequenced have several sources of natural variability. The overall genome arrangements for the human herpes viruses, for which details are available, are given in figure 1.1.

The completed sequence of HSV-1 (strain 17) was published by McGeoch et al., (1988). It consists of 152,260 nucleotides in each strand, with one overhanging residue at each end (Mocarski and Roizman, 1982) and has an overall G+C content of 68.3%. The genome can be considered to be subdivided into covalently joined long $_{(L)}$ and short $_{(S)}$ segments which are themselves composed of a central unique sequences (U_L and U_S) flanked by oppositely oriented repeats (TR_L and IR_L, TR_S and IR_S). Directly repeated a sequences of 400bp exist at the genomic termini and at least one copy of the a sequence is also found at the junction of the $_L$ and $_S$ segments (Davison and Wilkie, 1981, Roizman, 1979). The genome exists in four equimolar sequence orientations formed by the relative inversion of the long and the short sequences; one of these has been designated the prototypic orientation. The genome structure and composition of HSV-2

is very similar to that of HSV-1 albeit with a slightly higher G+C content.

Analysis of the VZV genome was commenced by Ludwig et al., (1972) who used isopycnic sedimentation to determine its G+C content to be 46%. Further analysis of the genome, (Dumas et al., 1980), using DNA isolated from infected cultures, suggested a molecular weight of $80x10^6$ (approximately 120kbp). The gross structure of the VZV genome was then investigated by restriction mapping (Dumas et al., 1981) and examination of annealed single stranded virion DNA molecules by electron microscopy (Ecker and Hyman, 1982; Straus et al., 1982; Gilden et al., 1982). These initial studies suggested that the VZV genome consisted of two segments, $_L$ and $_S$. The $_L$ segment was shown to be a unique sequence of 100kbp while the $_S$ segment consisted of a central unique portion of 5.4kbp flanked by inverted repeated sequences of 6.8kbp. Further analysis of restriction endonuclease digestion patterns showed half molar fragments of TR_S and IR_S suggesting the existence of two genome isomers formed by the inversion of the $_S$ segment relative to the $_L$ segment (Dumas et al., 1981; Straus et al., 1981,1982; Ecker and Hyman, 1982). More detailed analysis of the genomic termini and comparison with the $_{LS}$ joint by sequencing revealed that, in contrast with most herpes viruses, the VZV genome possessed no terminal redundancy (Davison, 1984), and that 88.5 bp inverted repeats were present flanking the $_L$ segment (Davison, 1984). Like HSV-1, each 3' end of the genome was shown to have a single unpaired residue, C at the $_L$ segment terminus and G at the $_S$ segment terminus. Finally this study demonstrated the presence of two minor genomic arrangements constituting 5% of the total and resulting from the inversion of the $_L$ segment from its prototypic orientation (Davison, 1984). Thus, the overall structures of VZV and HSV genomes are similar but VZV remains unique amongst herpes viruses thus far analysed in having unequal proportions of its sequence orientations.

A total of 14 restriction endonuclease maps of the VZV genome were published during the early 1980's (Dumas et al., 1981; Ecker and Hyman, 1982; Straus et al., 1982, 1983; Davison and Scott, 1983; Gilden et al., 1982; Mish ra et

al., 1984). These, along with epidemiological studies of virus isolates and studies of genome variability in vivo and in vitro, led to the demonstration of variability in the size of fragments derived from specific locations within the virus genome. Comparison of these with the sequence of the Dumas strain (Davison and Scott, 1986) showed that the variability mapped to three locations at which tandem direct reiterations of short G+C rich sequences were found. A fourth variable locus was found in the U_L sequence close to the $_{L-S}$ joint (Hondo et al., 1987); this contains 88bp direct repeats with three mismatches which are not G+C rich and are separated from each other by 24bp spacers. The role, if any, of these variable regions, which also occur in other herpes viruses in both coding and non-coding regions of the genome (Davison and Wilkie, 1981; Watson et al., 1981), is not clear.

The sequence of HCMV, though nearing completion, is as yet unpublished. It is larger than those of HSV-1, VZV and EBV, being approximately 230kbp with a G+C content of 56%. Its overall arrangement is similar to that of HSV, having long and short segments with inverted terminal repeats (Westphate et al., 1983), an equivalent to the a sequence, and 4 equimolar sequence orientations (Spaete and Mokarski, 1985).

EBV was the first herpes virus whose complete sequence was determined. The sequence, 172282 residues of base composition 59.9% G+C, was derived from the B95-8 strain (Baer et al., 1984). The arrangement of the genome shows considerable differences from that of HSV, having long and short unique sequences bounded by variable numbers of 540bp direct repeats at the termini and 3072bp direct repeats internally.

A much less complete picture is available for the gross structure of the HHV6 genome although preliminary results suggest that it contains approximately 160kbp with a base composition of 42% G+C it consists of a single unique region flanked by direct repeats and appears to be most closely related to HCMV (Lawrence et al., 1990).

1.2. Biology and Pathology of VZV Infections

1.2.1. VZV Infections *In Vivo*.

As noted above, VZV causes two main clinical conditions, chickenpox, a childhood exanthem, and shingles which follows reactivation.

Since chickenpox is a benign condition and lacks a good animal model (Provost *et al.*, 1987), knowledge of its pathogenesis comes largely from analogy with other viral exanthems (Gelb, 1985). Infection appears to occur through the mucosa of the upper respiratory tract or the conjunctivae with both air spread and direct transmission being possible as routes of infection (LeClair *et al.*, 1980; Gustafson *et al.*, 1982; Weller, 1983).

An incubation period of about 14 days occurs between primary infection and the appearance of vesicles. During this period the virus replicates at the initial site of infection and then spreads as a primary viraemia in the blood and lymph. The virus is taken up by the cells of the reticuloendothelial system and undergoes multiple cycles of replication which result in a secondary viraemia (Feldman and Epp 1979). This is associated with prodromal symptoms which are rapidly followed by the typical vesicles occurring in the skin, the mucous membranes and the parenchyma of almost every organ (Cheatam *et al.*, 1956, Johnson, 1940, Taylor-Robertson and Caunt, 1972). Vesicle formation is initiated by infection of the endothelial cells of capillaries allowing virus spread to the surrounding tissues. In the case of the skin this results in the infection of basal layers of the epidermis causing extracellular oedema, separation of the basal layer s from the unaffected stratum corneum and thus formation of the typical vesicle (Gelb, 1985). New vesicles occur in crops over a period of three days (Gold, 1966) after which, under normal circumstances, the immune response to the virus results in its clearance (Gershon and Steinberg, 1979, Feldman *et al.*, 1975, Rand *et al.*, 1977).

Complications of chickenpox fall into two categories. First, uncontrolled infection can lead to varicella pneumonia with widespread damage to the linings of small blood vessels in the lungs resulting in thrombosis and haemorrhage (Cheatham *et al.*, 1956). Second, aberrant

immune responses to the virus may result in meningoencephalitis, Guillian-Barre syndrome and Reyes syndrome, which can occur up to three weeks after the onset of the rash (Johnson, 1982, Johnson et al., 1985, Kennedy, 1987).

During the course of its primary infection VZV establishes a latent infection in the sensory ganglia of spinal nerves and the trigeminal nerve, as proposed initially by Hope-Simpson (1965). That this occurs at the vesicular stage of the infection is suggested by the finding that frequency of reactivation in a given dermatome correlates with the density of vesicles during the primary infection (Gelb, 1985, Kennedy, 1987). Evidence that the dorsal root ganglia are indeed the site of latency is argued for by the unilateral, segmental pattern of recurrence. However, direct evidence is lacking as, unlike HSV, VZV cannot be recovered from ganglia obtained randomly at autopsy (Price, 1986; Kennedy, 1987), though viral DNA has been detected in lysates of ganglia in the absence of clinical disease by Southern hybridisation (Gilden et al., 1983). Attempts to localise the site of VZV latency within the ganglia are more controvertial, with both neuronal (Hyman et al., 1983) and non-neuronal (Croen et al., 1988) sites being proposed. Both of these studies detected RNA by in situ hybridisation from many but not all areas of the genome, raising the possibility that at least partial reactivation had occurred as a result of the death or illness of the donor. These findings are in contrast to the single family of latency associated transcripts (LATs) mapped to the long terminal repeats of HSV-1 (Stevens et al., 1987, Wechsler et al., 1988). The significance of these differences between the HSV and VZV systems is not, as yet, clear.

While too little is known of the processes of the initiation and maintenance of latency to make meaningful comparisons between HSV and VZV, differences in reactivation are obvious at both clinical and histological levels. HSV reactivates to form a small lesion affecting a small part of a dermatome while VZV reactivation classically results in the appearance of vesicles throughout the dermatome. The histological picture of VZV reactivation is of acute

inflammatory changes throughout the ganglion affected, with neuronal death and resulting sensory loss (Zacks et al., 1964, Ghatak and Zimmerman, 1973), while there is reason to doubt whether neuronal death results from the reactivation of HSV (Price, 1986). These findings suggest reactivation of VZV results in a full lytic infection within the ganglion as well as at the epithelium, in contrast, no such evidence of infection throughout the ganglion exists for HSV reactivation.

The stimuli that result in clinically detectable reactivation also differ between the two viruses. HSV can be induced to reactivate by a variety of stimuli including exposure to UV light, fever, stress and hormonal changes (Wildy et al., 1982, Hill, 1985). By contrast reactivation of VZV is strongly associated with reduced immunity (Hope-Simpson, 1965, Schimpff et al., 1972). Indeed, there is evidence to support a model of frequent, subclinical, reactivations of latent VZV, in which clinical presentation is prevented by an effective immune response but the antigen released is sufficient to boost antibody levels and so mark the event (Luby et al., 1977). These alterations in humoral immunity could also be explained by reexposure to exogenous VZV which also occurs (Brundell et al., 1975, Gershon et al., 1984).

Investigation of the mechanism of latency and reactivation would obviously be facilitated by the availability of a animal model system which replicates the pathogenesis of VZV infection. Neither of the published systems, guinea pig (Myres et al., 1980, 1985, Walz-Cicconi et al., 1986) or common marmoset (Provost et al., 1987), show external signs of disease or evidence that they could be used for the investigation of latency.

1.2.2. Growth of VZV in Tissue Culture

Problems in developing systems for the investigation of VZV biology continue when the growth of the virus in tissue culture is considered. In common with its restricted host range in vivo, human and primate cells have been found to be most permissive for virus replication in tissue culture

(Grose and Brunell, 1978, Schmidt and Lennette, 1976, Caunt and Taylor-Robinson, 1964). However, even when permissive cells are infected, only low titres of cell free virus can be obtained (Taylor-Robinson and Caunt, 1972, Shiraki and Hyman, 1987). In contrast, virus infected cells can be used to propagate infections at high efficiency (Rapp, 1964). The dearth of infectious cell free virus is hard to explain as large numbers of apparently mature virions are found associated with the surface of cells late in infection. Indeed, studies comparing pfu with the numbers of particles of normal morphology in sonicated samples of infected cells have determined a ratio of approximately 10^6:1 (Shiraki and Takahashi, 1982, Gelb, 1985). Coat lability (Cook and Stevens, 1968), lysosomal enzymes, (Gershon et al., 1973) and faulty maturation in tissue culture (Becker et al., 1965) have been proposed to explain this phenomenon. Even when infectious cell free virus is obtained, either from tissue culture or directly from vesicles, the rate at which infection proceeds is less than that seen with infected cells (Caunt and Taylor-Robinson, 1964, Schmidt and Lennette, 1972) suggesting that factors related to the infected cells as well as the virus itself, may be relevant to the in vitro phenotype of the virus.

Attempts have also been made to investigate the interaction of VZV with neurones using cell culture systems (Wigdahl et al., 1986, Vafai et al., 1988, Merville-Louis et al., 1989). These workers came to different conclusions regarding both the ability of VZV to undergo lytic infection and the transcripts and proteins produced during the infective process. The discrepancies were presumably related to differences in the sources of the cells and the culture systems used. Thus the relevance of any or all of these systems to viral infections of humans awaits further evaluation.

1.3. Molecular Biology of VZV

1.3.1. The VZV Genome.

The derivation of the sequence of the VZV genome

(Davison and Scott, 1986) marked a turning point in the study of the virus. Prior to this study the information available regarding products induced by virus infection was restricted to two main groups, first, activities of pyrimidine deoxynucleoside kinase (Doberson et al., 1976), DNA polymerase (Miller and Rapp, 1977) and endonuclease (Cheng et al., 1979) enzymes had been detected. Second three major families of glycoproteins had also been defined using monoclonal antibodies (Davison et al., 1986; section 1.5.3.4.). In addition, several attempts had been made to enumerate virus specified proteins using SDS-PAGE (Wolff, 1978; Asano and Takahashi, 1979, 1980; Shemer et al., 1980; Shiraki et al., 1982).

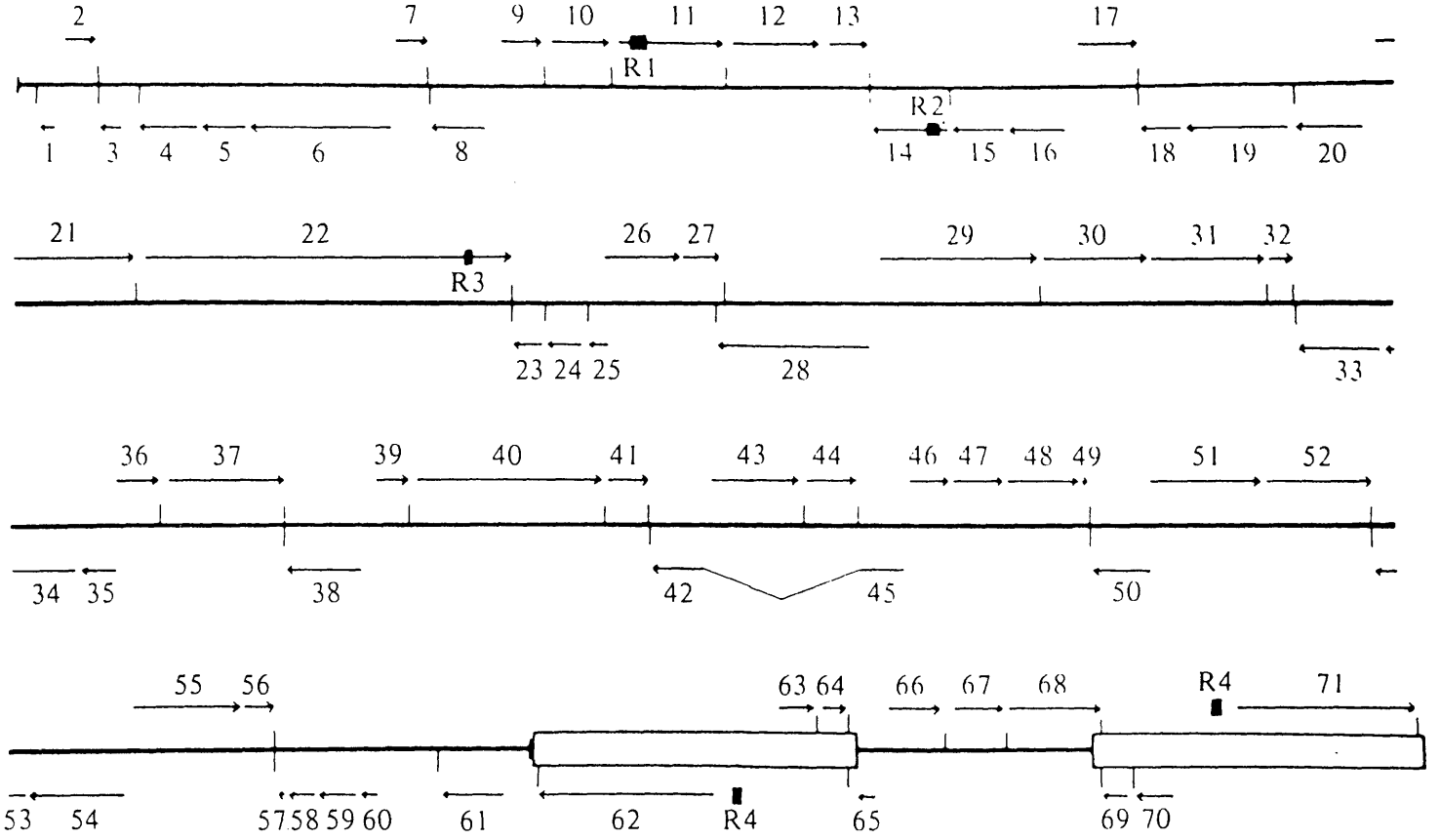
Analysis of the complete sequence of VZV (Davison and Scott, 1986) resulted in the identification of 71 orfs in the VZV genome. Of these, 65 are unique and three are present in each of the repeated sequences flanking the short unique region (figure 1.2.; table 2.). Due to the similar genome arrangements in VZV and HSV, these data have provided information about the functions of over one third of the predicted VZV orfs (Table 2). Additional information came from comparison of VZV sequence data with that in the NBRF database. This revealed similarities between VZV orf13 and known thymidylate synthetase enzymes and between orf66 and known protein kinases. Only the latter of these is also present in the HSV-1 sequence (McGeoch and Davison, 1986). Since the publication of the VZV sequence, new data have come to light which have allowed allocation of functions to five VZV orfs probably involved in DNA replication. These are homologues of the HSV proteins which, along with the DNA polymerase and the major DNA binding protein, have been shown to be necessary and sufficient for origin dependent DNA replication (Challberg, 1986). In addition orf61 has been shown to have a small but significant degree of homology with the HSV IE gene 1 (Perry et al., 1986), orf59 has been shown to bear strong similarities to the recently recognised uracil DNA glycosylase (Worrad and Carradona, 1988; Mullaney et al., 1989) and orf47 to other protein kinase (Smith and Smith, 1989).

Three studies have reported attempts to map RNA transcripts to individual orfs (Maguire and Hyman, 1986;

Figure 1.2. SUMMARY OF VZV GENE LAYOUT

The genome is represented 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 (TR_L and IR_L). ORFs 1 to 71 are illustrated as arrows against the appropriated strand, and correspond to the protein coding regions shown in Table 1. Light vertical lines indicate potential polyadenylation sites in the appropriate strand. The locations of the 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.

Taken directly from Davison and Scott, (1986) with permission.


2
7
9
10
11
12
13
17
R1
R2
1
3
4
5
6
8
14
15
16
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19
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R3
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64
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67
68
R4
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54
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59
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61
62
R4
65
69
70

Table 1.2.

ORF*	Codons	Mol. wt.+	Function@
1	108	12103	
2	238	25983	
3	179	19149	
4	452	51540	homologue of HSV-1 IE63
5	340	38575	
6	1038	122541	DNA replication
7	259	28245	
8	396	44816	dUTPase
9	302	32845	
10	410	46375	homologue of HSV-1 Vmw65
11	819	91825	
12	661	74269	
13	301	34531	thymidylate synthetase
14	560	61350	glycoprotein (gpV) gC
15	406	44522	
16	408	46087	DNA replication
17	455	51365	
18	306	35395	ribonucleotide reductase
19	775	86823	
20	483	53969	
21	1038	115774	
22	2736	306325	
23	235	24416	
24	269	30451	
25	156	17460	
26	585	65692	
27	333	38234	
28	1194	134041	DNA polymerase
29	1204	132133	major DNA-binding protein
30	770	86968	
31	868	98062	glycoprotein (gpII) gB
32	143	15980	
33	605	66043	
34	579	65182	
35	258	28973	
36	341	37815	deoxynucleoside kinase
37	841	93646	glycoprotein (gpIII) gH
38	541	60395	
39	249	27078	
40	1396	154917	major capsid protein
41	316	34387	
42	395	82752	
45	352		
43	676	73905	
44	363	40243	
46	199	22544	
47	510	54347	protein kinase
48	551	61268	exonuclease
49	81	8907	
50	435	48669	
51	835	94370	DNA replication
52	771	86343	DNA replication
53	331	37417	
54	769	86776	
55	881	98844	DNA replication
56	244	27166	
57	71	8079	

58	221	25903	
59	305	34375	uracil DNA glycosylase
60	159	17616	
61	467	50913	homologue of HSV-1 IE110
62/71	1310	139989	homologue of HSV-1 IE175
63/70	278	30494	homologue of HSV-1 IE68
64/69	180	19868	
65	102	11436	
66	393	43677	protein kinase
67	354	39362	glycoprotein (gpIV) gI
68	623	69953	glycoprotein (gpI) gE

* Data largely taken from Davison and Scott, (1986)

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

@ References to appropriate DNA sequence data from HSV or other organisms which confirm the assignment of VZV gene functions are largely given in Davison and Scott, (1986). Functions characterised since then are referenced in the text.

Ostrove et al., 1985; Reinhold et al., 1988). In the most recent of these, a total of 78 transcripts were identified and their direction mapped by northern blotting. Sizes ranged from 0.8kbp to over 8.0kbp and the probes used represented over 99.7% of the genome. In a few cases it was possible to map transcripts to individual orfs, however in most cases lack of resolution in the mapping made the allocation of transcripts to specific orfs impossible. In yet other cases transcripts mapped appeared not to correlate with any orf predicted from the sequence. More detailed mapping and eventually nuclease S1 analysis will be required to define the locations of transcripts. This has been achieved for only two VZV orfs, number 61, the major IE gene, (this thesis) and number 36, the dPyK, (Davison and Scott, 1986).

Along with descriptions of the functions of VZV proteins, studies into the control of their synthesis has suffered greatly from the lack of high titre cell free virus with which to carry out infections. However, it has been assumed that gene regulation in VZV occurs in a manner similar to that described in HSV and other herpes virus infections, that is, in the form of a cascade of three broad groups of proteins, immediate early (IE), prior to viral protein synthesis, early (E), following viral protein synthesis but prior to DNA synthesis, and late (L) following genome replication. This classification of genes is not absolute. For example, in HSV the large subunit of ribonucleotide reductase is regulated as an early protein. However, inhibition of protein synthesis, and thus early gene expression, does not prevent its transcription (Honess and Roizman, 1974; DeLuca et al., 1984). A similar situation occurs with regard to the separation of E and L gene expression by DNA replication.

General functions can be ascribed to each temporal class of HSV proteins, IE genes fulfil regulatory roles, E genes provide the machinery for viral DNA synthesis and L genes encoding the fabric of the virus. Evidence that this general plan of genome expression and functions holds for VZV comes, first, from sequence analysis, which reveals many similarities between the genes encoded by the two viruses. In addition, two attempts to analyse VZV IE proteins using

cycloheximide block and release in the presence of actinomycin D have been made (Lopetegui et al., 1985; Shiraki and Hyman, 1987). The results concurred in detecting a subset of VZV proteins synthesised under the "IE" conditions, suggesting similarities to the HSV system, and in the molecular weights of some of the proteins produced. However, there were discrepancies as to the size and number of the minor species present.

1.3.2. Immediate Early Genes of VZV

1.3.2.1. VZV orf62.

VZV orf62 encodes a protein of predicted molecular weight 140,000 (140K) that bears strong sequence similarity to HSV-1 IE gene 3, which encodes the protein Vmw175 (McGeoch et al., 1986; Davison and Scott, 1985) and the equine herpes virus 1 (EHV-1) major IE gene, which encodes a protein of predicted molecular weight 155,000 (Grundy et al., 1989). These homologies are localised to two regions within the coding sequences, region 2 and region 4, amino acids 468-641 and 736-1148 of the VZV sequence, respectively (McGeoch et al., 1986; Grundy et al., 1989).

The functions of the HSV protein, Vmw175, have been studied in detail due to its central role in the transcriptional programme. Conditional HSV-1 mutants with lesions in IE gene 3, notably tsK, fail to synthesise E and L proteins at non-permissive temperature (Marsden et al., 1976; Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1978, 1980) and over synthesise IE proteins and Vmw136 (Marsden et al., 1976; Preston, 1979a). Upshift of the virus at later stages of infection returns the virus to this phenotype (Preston, 1979b; Watson and Clements. 1980) suggesting that the requirement for Vmw175 continues throughout the lytic cycle. These and other experiments suggest that major functions of this polypeptide are both the control and maintenance of the transition from IE to E and L phases of transcription, and the regulation of its own transcription. In this context it is interesting to note that Vmw175 binds to the hexanucleotide ATCGTC which is

found at its own transcriptional start site (Faber and Wilcox, 1986).

Functional correlations between 140K and Vmw175 have also been noted. Analysis of the transactivation of both the HSV glycoprotein D and the rabbit beta globin promoters in short term transfection assays suggested that 140K was a more efficient transcriptional activator than Vmw175 (Everett and Dunlop, 1984; Everett, 1984). Furthermore, Vero cells that contain integrated copies of orf62 (F114 cells) complement the growth of HSV-1 ts mutants whose mutations are in the coding sequences for Vmw175. Interestingly, the transcription of 140K from F114 cells could be stimulated by superinfection with HSV-1, an effect that occurred even in the presence of cycloheximide suggesting that, like HSV IE genes, (see section 1.4.), the transcription of orf62 could be stimulated by a component of the HSV virion, (Felsner et al., 1988).

1.3.2.2. VZV orf4.

This orf is predicted to encode a protein of molecular weight 51,540 (Davison and Scott, 1986). Its sequence shows similarities to that of the HSV IE protein Vmw63 which is encoded by HSV IE gene 2 (McGeoch et al., 1988). Analysis of the role of this HSV protein using both ts mutants (Sacks et al., 1985) and deletions (McCarthy et al., 1989) revealed it to be essential for viral replication in tissue culture and that its absence resul ted in a severe restriction of the production of 'true' L proteins (Sacks et al., 1985; McCarthy et al., 1989). Analysis of the function of Vmw63 in transient expression assays has yielded somewhat contradictory results but it seems that the protein acts in concert with Vmw175 and Vmw110 to regulate, both positively and negatively, the expression of subsets of viral genes (Everett, 1986; Rice and Knipe, 1988; Sekulovich et al., 1988) in apparent agreement with the phenotypes of virus mutants (Sacks et al., 1985; McCarthy et al., 1989). Homologues of orf4 have also been detected in two gamma herpes viruses, EBV (Davison and Taylor, 1987) and herpes virus saimiri (Nicholas et al., 1988). The products of these orfs act both independently and in concert with the

product of the EBV gene BZLF1 to stimulate expression from a range of both viral and cellular promoters (Nicholas et al., 1988; Chevallier-Greco et al., 1986). Finally, recent evidence suggests that the product of orf4 can act in short term transfection assays to stimulate expression from the VZV dPyK promoter and from other viral and cellular promoters (Inchauspe and Ostrove, 1989).

1.3.2.3. Orf63

This orf is predicted to encode a protein of molecular weight 30,494 and shows sequence similarities to the HSV IE protein Vmw68. The functions of neither of these proteins is known. A mutant of HSV-1 has been constructed which lacks the carboxyterminal third of Vmw68 (Post and Roizman, 1981). It exhibits poor growth in tissue culture, reduced expression of at least one L gene in some cell lines and was not neurovirulent in mice (Sears et al., 1985).

1.3.2.4. Orf61.

This orf encodes a homologue to HSV-1 IE gene 1 which encodes the protein Vmw110. Sequence similarities between the proteins are confined to a region in the amino terminus of the protein which has the potential to encode a zinc finger domain (Perry et al., 1986; Berg, 1986). The HSV protein is not strictly essential for viral replication in tissue culture. Virus mutants that lack both the entire protein, dl1403 (Stow and Stow, 1986) and the zinc finger domain alone, FXE (Everett, 1989), exhibit a similar phenotype growing efficiently at high multiplicity of infection (moi) and in a very restricted fashion at low moi especially on slow growing cell types (Stow and Stow, 1986; Everett, 1989). This suggests that Vmw110 has a role early in infection in committing the viral genome to the lytic cycle especially in situations where low copy numbers of the genome are present. In transient expression assays in cell culture Vmw110 acts as a transactivator of early gene expression both independently and in synergy with Vmw175 (Everett, 1984b; O'Hare and Hayward, 1985a and b; Quinlan and Knipe, 1985; Mavromara-Nazos et al., 1986). Mutational

analysis of the protein showed that these two roles were separable and that the zinc finger domain was essential for independent transactivation (Everett, 1987, 1988a). Interestingly, in view of the specific region found to be conserved between the HSV and VZV proteins, VZV coinfection rescues the HSV mutant dl1403 (Stow and Stow, 1989). No evidence exists that this phenomenon is related to orf61, indeed coinfection with HCMV, which lacks a Vmw110 homologue, also rescues the mutant.

It should be emphasised that no definitive evidence exists that any of the VZV orfs mentioned above is regulated as an IE gene, though combining the expression evidence of Shiraki and Hyman, (1987) with sequence comparisons and functional studies suggest that orf62, orf4 and orf63 represent good candidates for IE genes with the situation regarding orf61 being rather less clear.

1.3.3. Regulation of Early Gene Expression.

As yet, due to the inability to induce co-ordinate infections with VZV, there is no direct evidence as to the presence of a distinct class of E genes, that is, those produced following protein synthesis but not dependent on DNA replication.

Indirect evidence suggests that this class of proteins does exist. Sequence analysis reveals clear homologues of many of the E genes encoded by the HSV genome. What is known about the function of VZV IE genes (Everett, 1984; Everett and Dunlop, 1984; Felsner et al., 1987; Inchauspe and Ostrove, 1989) suggests that orf62 and orf4 function as transactivators, and one would predict that their targets would be E and L genes. This supposition is reinforced by very preliminary studies on the VZV dPyK control region which show that this promoter has a TATA box which alone is sufficient to allow transactivation by the VZV IE gene homologues (Inchauspe and Ostrove, 1989).

The control of E gene expression in HSV has been studied using both transient expression assays and viruses containing mutations in the regulatory sequences of the E genes (Everett, 1983, 1984b; Coen et al., 1986). These

studies defined several functional elements including the cap site, TATA box, GA rich sequences, Spl binding sites and CCAAT box motifs to be important for E gene transcription. None of these was shown to be solely responsible for the stimulation of expression by IE gene products, though experiments with mutants returned to virus suggested that the TATA box of the dPyK gene was of primary importance (Coen et al., 1986).

While the model of HSV gene expression predicts that E genes require IE gene products to stimulate their expression other findings question this simplistic viewpoint. The demonstration that the rabbit beta-globin gene was regulated as an early gene in transient expression assays and when integrated into the virus genome but not when its natural chromosomal environment in rabbit kidney cells (Everett, 1983, 1984b; Smiley et al., 1982), and the ability of the HSV TK promoter to function autonomously in transient expression assays but apparently not in the viral genome, raises the possibility that mechanisms other than cis-acting sequences are important in the control of E gene expression in the context of the viral genome.

1.3.4. Control of Late Gene Expression.

No direct evidence is available that defines a separate class of VZV L genes, dependent for their full expression on the replication of viral DNA.

HSV-1 again provides the model for L gene control. The available evidence suggests that the expression of 'true' L genes, those whose expression cannot be detected prior to DNA replication, such as US11, depends only on the presence of a TATA box and a linked, functional, origin of replication (Johnson and Everett, 1986b; Johnson et al., 1986). This finding was confirmed by the conversion of an E gene, gD, to L gene regulation, as assayed in short term expression assays, by the deletion of sequences upstream of its TATA box and linkage to an HSV origin of replication. The other category of L genes, known as 'leaky' L genes, produce proteins that are detectable prior to DNA replication but require this event for their full

expression. The major capsid protein Vmw155, is an example of this category. Analysis of virus mutants with mutations in the IE genes encoding Vmw63 or Vmw68 suggest that these IE proteins are involved in the control of L gene expression (Sears et al., 1985; Sacks et al., 1985) and analyses using transient expression assays suggest that other IE genes can also exert an effect (Dennis and Smiley, 1984; DeLuca et al., 1985; Mavromara-Nazos et al., 1986; Everett, 1986). The mechanism by which DNA replication induces L gene expression is unknown.

1.3.5. VZV Encoded Enzymes.

1.3.5.1. Pyrimidine Deoxynucleoside Kinase.

The presence of novel thymidine kinase, deoxycytidine kinase and bromodeoxycytidine kinase activities in cells infected with VZV was first detected by Doberson et al., (1976). They also determined that the activities co-purified with a column fraction of approximate molecular weight 70,000. These findings were confirmed and extended by the demonstration that the activities were due to a single protein species (Hackstadt and Mallavia, 1978). Analysis of the protein under denaturing conditions revealed a single species with a molecular weight of 35,000, suggesting that the functional form of the enzyme was a dimer (Lopetegui et al., 1983; Shiraki et al., 1985). In common with other herpes virus TKs, and in contrast to cellular TKs, the VZV enzyme functions as a deoxycytidine kinase (Jamieson and Subak-Sharpe, 1974; Cheng et al., 1979) and as a thymidylate kinase (Chen et al., 1979; Chen and Prusoff, 1978). However, the properties of the VZV enzyme were found to differ from those of HSV-1 in its heat stability and its ability to phosphorylate deoxycytidine in preference to thymidine (Cheng et al., 1979).

The location of the gene encoding the dPyK activity was mapped between 0.50 and 0.52 map units by Sawyer et al., (1986) who investigated the transformation of LTK^- cells to a TK^+ phenotype with restriction endonuclease fragments. They also found that for a given TK activity a much higher

copy number of VZV genes was present in transformed cells compared with cells transformed with HSV-1 TK. A possible explanation for this finding was provided when the gene and its upstream sequences were sequenced and the 5' end of the mRNA mapped. This revealed the presence of two initiation codons closely followed by termination codons in the 5' untranslated region of the mRNA. These small orfs were in different reading frames from that of the dPyK and may represent a mechanism to reduce translational efficiency. This finding might explain the finding of Sawyer et al., (1986) who found that many more copies of the VZV dPyK gene were required to produce the same level of TK activity in LTK^- cells transformed with a fragment containing VZV orf36 compared with those transformed with an equivalent HSV fragment.

Herpesvirus TKs differ structurally from their cellular counterparts in that their functional form is a dimer (Shiraki et al., 1985) while vaccinia virus TK, which is similar to cellular TKs, functions as a tetramer (Kit et al., 1985). The enzymes' sequences reflect these functional differences. Those herpes virus TKs for which sequence data are available (McKnight, 1980; Wagner et al., 1981; Swan and Galloway, 1983; Otsuka and Kit, 1984; Baer et al., 1984; Davison and Scott, 1986) have no recognisable similarities to the TKs from several vertebrates and pox viruses (Boyle et al., 1987) beyond a region common to all enzymes which use ATP (Otsuka and Kit, 1984; Walker et al., 1982; Gentry, 1985; Fry et al., 1986). Comparison of the herpes virus TK sequence with that of yeast thymidylate kinase (Jong et al., 1984), the only sequence data available for that enzyme, demonstrated a greater degree of similarity than between herpes virus and cellular TKs (Robertson and Whalley, 1988). This led the authors to suggest that herpes virus TKs may have evolved from cellular enzymes other than TKs, possibly thymidylate synthetase or deoxycytidine kinase.

1.3.5.2. Thymidylate Synthetase.

Another enzyme involved in thymidine metabolism is thymidylate synthetase (TS), which converts deoxyuridine monophosphate to thymidine monophosphate. It was detected

in the VZV sequence by its considerable homology with other TS enzymes from bacteriophage to man, and is not present in the HSV-1 sequence (Davison and Scott, 1986). Confirmation of the sequence data came with the expression of the orf in an E. coli strain whose own TS had been deleted (Thompson et al., 1987). Cells expressing the putative VZV TS were able to grow in the absence of thymidine and released tritiated water from 2-deoxy[5-^{3}H]uridylate. The enzyme could also be specifically labelled with 5-fluoro-2-deoxyuridine[^{32}p]monophosphate and was found to have a molecular weight of 32,500 on SDS-PAGE.

Thus VZV can produce thymidylate de novo using the TS and by thymidine salvage using the dPyK. The reasons for the presence of both enzymes in the genome, especially in view of the absence of a TS from HSV, are not clear. Nevertheless, the presence of this enzyme in VZV infected cells might provide a target for antiviral chemotherapy.

1.3.5.3. Protein Kinases.

As mentioned in section 1.3.1., comparison of the sequence of orf66 with those of non-herpes proteins led to the identification of both it and the HSV-1 gene US3 as possible protein kinases (McGeoch and Davison, 1986). Many of the conserved regions within these proteins align with the regions of greatest similarity in several eukaryotic protein kinases, particularly those of the serine-threonine kinase family. These results were of particular interest in view of the reports from various laboratories of protein kinase activity associated both with HSV virions (Rubenstein et al., 1972; Lemaster and Roizman, 1980) and with cells infected by HSV-1 and the closely related pseudorabies virus (Blue and Stubbs, 1981; Purves et al., 1986). The evidence available regarding the characteristics of the kinases detected were somewhat contradictory. Purves et al., (1986), described a kinase present in infected cells, but not virions, that is expressed as an early protein and phosphorylated viral and other polypeptides on serine and threonine residues. Roizman's group (Lemaster and Roizman, 1980), in contrast, described a kinase, present in the capsid/tegument structure of virions which phophorylated

only virion proteins. The resolution of which of these activities corresponds to the kinase encoded by US3 has come from work by Frame et al., (1987), who demonstrated antigenic identity between the kinase isolated by Purves et al., and the product of US3 using an anti-peptide monoclonal antibody to the C-terminus of US3, and Purves et al., (1987), who demonstrated that an HSV-1 mutant, from which the majority of gene US3 had been deleted, failed to induce a novel protein kinase. This virus was, however, able to grow normally in tissue culture (Longnecker and Roizman, 1987), suggesting that the activity of this kinase is non-essential in this situation.

Although no direct evidence exists that VZV orf66 encodes a protein kinase the degree of homology between this gene and US3 is strongly suggestive. In this connection Montalvo and Grose (1987), have reported a virus-induced polypeptide resolving to 50,000 daltons on SDS-PAGE which phosphorylates GpI, the major VZV glycoprotein, on serine and threonine residues but has no effect on other VZV glycoproteins. This size is consistant with the protein being the product of orf66, which has a predicted molecular weight of 43,677, but further studies will be required to characterise the protein and other possible substrates further.

Finally, another gene, conserved amongst HSV, VZV and EBV, has been predicted to be a protein kinase. No information is yet available as to the functions of the products of these genes, UL13 in HSV, orf47 in VZV and BGLF4 in EBV, but their amino acid sequences make it likely that they are serine/threonine rather than tyrosine kinases (Smith and Smith, 1989).

1.3.5.4. Glycoproteins

Sequence analysis of the VZV genome revealed five open reading frames with sequence characteri stics of glycoproteins (Davison and Scott, 1986), that is, a hydrophobic signal sequence close to the amino terminus and a carboxy terminal region comprising a more extensive hydrophobic membrane spanning portion followed by a basic, cytoplasmic, portion. These have now all been shown to

specify glycoproteins, the nomenclature of which was standardised at the 1985 Herpes Virus workshop (Davison et al., 1986). The gpI family consists of 2-4 proteins ranging in molecular weight from 45,000-100,000 encoded by orf68 (Ellis et al., 1985). They are the most abundant and immunogenic of the VZV envelope glycoproteins and stimulate the production of complement dependent neutralising antibodies (Edson et al., 1985a). Analysis of gpII on SDS-PAGE revealed a major species of molecular weight 140,000 on non-reducing SDS-PAGE but 65,000 on reducing gels suggesting that this protein consists of a disulphide linked heterodimer (Vafai et al., 1983; Grose et al., 1984). It is encoded by orf31 (Keller et al., 1986a) and shows homology to HSV-1 gB both on sequence analysis and using monoclonal antibodies (Edson et al., 1985b; Kitamura et al., 1986). The third most abundent VZV glycoprotein, gpIII, migrates with a molecular weight of 105,000 to 130,000 on SDS-PAGE. Using a partial amino acid sequence of the purified protein, Keller et al., (1986b) showed that it is encoded by orf37. Antipeptide antibodies were used to characterise the product of orf67 as gpIV, a group of minor glycoproteins of molecular weight 45,000 to 55,000 (Davison et al., 1985). Again molecular analysis has been used to identify the product of orf14 as gpV by its expression in vaccinia virus. This construction resulted in the production of specific glycoprotein products of molecular weight 85,000 to 95,000 (Kinchington et al., 1986)

1.3.6. DNA Replication.

Little or no direct evidence is available relating to the overall scheme of VZV DNA replication. However, the following scheme has been proposed using HSV-1 as a model (Davison, 1984). On entering the cell nucleus, the genome circularises, forming two $_{L}$-$_{S}$ joints, one pre-existing and one new. A phase of limited replication ensues during which recombination between the repeated sequences flanking the $_{L}$ and $_{S}$ sequences can occur. This is followed by a major phase of replication to form long molecules consisting of head to tail concatemers of the genome, probably by a rolling circle mechanism. Unit length genomes are then

produced by site specific cleavage which usually results in the major genomic configuration but occasionally the minor form is produced. This unequal distribution of isomers suggests that part of the signal for genomic cleavage is situated in the U_L region close to the normal site of cleavage and part is located in one or both of the repeated sequences that bound the L and S segments. Recognition of the signals present in repeated sequences alone would produce the minor genome configuration while recognition of the full sequence would generate major configuration.

Evidence for this scheme of replication comes again from comparisons with the HSV-1 system. HSV-1 encodes three origins of replication, one situated in both IR_S and TR_S and one in U_L (Frenkel et al., 1976; Jean et al., 1977; Rixon and Ben-Porat, 1979; Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). Any one of these origins can be deleted without affecting viral replication (Longnecker and Roizman, 1986; Polvino-Bodinar et al., 1987). OriS consists of a 90 bp fragment containing a 45 bp palindrome with 18 centrally located A or T residues flanked by G+C rich tracts, and is located between the 5' ends of IE genes 3 and 4/5 (Stow, 1982; Stow and McMonagle, 1983). OriL is located between the genes encoding the DNA polymerase and the major DNA binding protein and is similar in structure to OriS although it has a rather larger, 72 bp, palindrome (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller et al., 1985).

The VZV origins of replication are found in an equivalent position in the TR_S and IR_S to HSV oriS. They consist of a 45 bp palindrome with 33 centrally located A and T residues. On the 5' side of the IR_S palindrome the sequence CGTTCGCACTT, also found in HSV-1 origins, is present, and two further copies of this motif are found further 5' to th e VZV origin. Interestingly, plasmids encoding the VZV origin of replication can be stimulated to replicate by superinfection with both VZV and HSV-1 (Stow and Davison, 1986), though HSV induced replication was about 5% of the level of similar constructs encoding HSV-1 OriS.

Not surprisingly, given these sequence and functional similarities, VZV encodes homologues of all seven of the HSV-1 proteins shown by Challberg et al., (1986) to be necessary and sufficient for origin-dependent replication.

Two of these genes encode the viral DNA polymerase and the major DNA binding protein, orf28 and orf29 in the VZV genome. The UL42 gene product, VZV orf16, has been identified as a previously detected DNA binding factor found in extracts of HSV-1 infected cells (Parris et al., 1988). Recently presented evidence suggests that the UL9 gene product, VZV orf51, is a sequence specific origin binding protein (Olivo et al., 1988; Weir et al., 1989) and that the product of UL5, VZV orf55, is a component of the DNA helicase activity found in virus infected cells (Weir et al., 1989). In addition, many of the other proteins involved in DNA replication in HSV such as dUTPase (Wohl and Frenkel, 1980; Preston and Fisher, 1984), thymidine kinase (Kit and Dubbs, 1963; Jamieson et al., 1974), ribonucleotide reductase (Coen, 1972; Dutia, 1983), alkaline exonuclease (Keir and Gold, 1963; Preston and Cordingley, 1982), and uracil DNA glycosylase (Mullaney et al., 1989) also have homologues in VZV.

1.4. Regulation of Herpesvirus IE Genes.

1.4.1 Cis-acting Sequences

The initial evidence that the IE genes of HSV-1, in common with those of other viruses, were controlled by potent regulatory sequences came from experiments in which the synthesis of viral proteins was prevented by the use of metabolic inhibitors or viral ts mutants (Kozak and Roizman, 1974; Clements et al., 1977; Preston, 1979; Watson et al.,1979; Anderson et al., 1980; Dixon and Schaffer, 1980; Watson and Clements, 1980). This treatment resulted in the rapid accumulation of large amounts of IE mRNAs, which were translated into high levels of IE protein upon removal of metabolic inhibitors.

The nature of the IE regulatory signals was initially investigated by Post et al., (1981). They used the coding sequences of HSV-1 TK as a reporter gene under the control both of its own upstream sequences, which result in its regulation as an E gene, and those of IE gene 3. These constructs were used to convert TK^- HSV-1 mutants to TK^+. Growth of the viruses under IE conditions, using cycloheximide block and actinomycin release, resulted in detectable TK activity only when the reporter gene was regulated by IE upstream sequences. This showed that 5' flanking sequences were sufficient to determine the regulatory characteristics of IE gene expression. A further series of experiments were presented in which the IE 3-TK construct was used to produce a biochemically transformed cell line containing the chimeric gene. Superinfection of this cell line with HSV-1 resulted in stimulation of TK gene transcription even when viral protein synthesis was inhibited. This result distinguished HSV-1 from other viruses studied in implicating a component of the virion in stimulating expression from the IE regulatory sequences. This virion component was later identified as Vmw65, also termed VP16, the product of gene UL48, and is a major component of the virus tegument (Campbell et al., 1984; Section 1.4.2.).

These data were extended by the demonstration that the regulatory sequences of IE gene 3 could be divided into two

functional parts (Mackem and Roizman, 1982b). One of these was a promoter region which, like the wild type TK regulatory signals, was sufficient to transform TK^- cells and viruses to a TK^+ phenotype. The other was a distal regulatory element which, when added to either promoter domain, converted the expression of TK genes to an IE phenotype in both cells and viruses.

Further studies confirmed and expanded these initial observations by showing that the findings held both for all other IE genes in both HSV-1 and HSV-2 (Mackem and Roizman, 1982a,b,c; Cordingley et al., 1983; Preston et al., 1984; Mosca et al., 1985; Gaffney et al., 1985) and when other assay systems such as microinjection of Xenopus oocytes and short term transfection assays were used (Cordingley et al., 1983; Lang et al., 1984; Preston et al., 1984; Preston and Tannahill, 1984; Kristie and Roizman, 1984).

Sequence comparisons, together with more detailed deletion analysis, revealed the presence of conserved cis-acting sequences in the regulatory regions of all of the IE genes of HSV-1 and HSV-2. Of these, an AT rich motif, TAATGARAT (R=purine), was present in at least one copy in all IE control regions and appeared to be essential for the response to the virion factor (Mackem and Roizman, 1982a,b,c; Cordingley et al., 1983; Preston et al., 1984; Lang et al.,1984; Bzik and Preston, 1986; O'Hare and Hayward, 1985b). A 20-mer oligonucleotide containing this sequence was sufficient to confer responsiveness to the virion component when inserted, into a plasmid, in either orientation, upstream of a HSV IE promoter-CAT gene construct (Gaffney et al., 1985). Stimulation of transcription was increased by the presence of multiple copies of the oligonucleotide.

Sequences flanking the TAATGARAT motif have been shown to modify the response to Vmw65. In particular, G+A rich sequences can supplement the activity of TAATGARAT motifs, and were also found to convert an otherwise nonfunctional but related sequence, TAACGAGGAAC, to virion responsiveness (Bzik and Preston, 1986). However, these G+A sequences were not found to mediate significant independent activity. Similar results were reported by Triezenberg et al., (1988b), however they placed more emphasis on the importance

of the G+A rich sequences which, they suggested, would mediate stimulation by the virion factor in the absence of a TAATGARAT motif. Further studies will be required to resolve these differences.

In addition to the promoter and virion factor responsive elements present upstream of all IE genes, a distinct enhancer-like element was found in the intergenic region between IE genes 3 and 4/5 (Lang et al., 1984; Preston and Tannahill, 1984). This was found to be active in the absence of Vmw65 and to stimulate activity from linked promoters in an orientation independent fashion and from a considerable distance (at least 1000bp) upstream. However, unlike other enhancer sequences, the HSV fragment appeared not to function from a position downstream of the reporter gene (Preston and Tannahill, 1984). The crucial components of the enhancer-like element appear to be two GC-rich Spl binding site consensus sequences and the G+A rich motif described above (Bzik and Preston, 1986).

1.4.2. Virion Trans-Inducing Factor

As detailed above, initial experiments to characterise the regulatory sequences of HSV IE genes suggested that a component of the virus particle was involved in the stimulation of IE gene expression (Post et al., 1981). The location of the transinducing factor within the virion was investigated using the HSV-1 mutant, tsB7, which fails to uncoat at non-permissive temperature. This mutant retained its ability to stimulate the expression of HSV IE constructs at non-permissive temperature, suggesting that the stimulatory component was located external to the virus nucleocapsid (Knipe et al., 1981; Batterson and Roizman, 1983). The protein responsible for this effect was identified by Campbell et al. (1984), who co-transfected cloned restriction fragments of HSV-1 DNA with an IE-TK reporter plasmid and assayed for stimulation of TK expression. Having isolated a fragment capable of stimulating expression they located the gene responsible by subcloning and immunoprecipitation studies and identified it as that encoding Vmw65. This protein was known to be a

major tegument phosphoprotein, estimated to be present at approximately 1000 molecules per virion (Heine et al., 1974; Marsden et al., 1978). Protein Vmw65 is essential for virion assembly since the mutation in ts2203 has been mapped to this gene (Ace et al., 1988). This mutant fails to assemble virus particles at the non permissive temperature (V. G. Preston, pers. comm.).

Sequencing of the fragment encoding Vmw65 (Pellett et al., 1985; Dalrymple et al., 1985) revealed an orf with the potential to encode a polypeptide of predicted molecular weight 50,342, which was homologous to a VZV gene product represented by orf10. The most notable feature of the primary structure of the HSV orf is the presence of an 78 amino acid carboxy-terminal region distinguished by a high frequency of acidic residues giving it an overall charge of -19 at physiological pH. This carboxy-terminal "acidic tail" is absent from VZV orf10. Recent studies have revealed the importance of acidic domains in the function of a group of eukaryotic and prokaryotic transcriptional activators including Vmw65 (Hope and Struhl, 1986; Gill and Ptashne, 1987; Sadowski et al., 1988; Triezenberg et al., 1988a; Greaves and O'Hare, 1989). These results suggest that Vmw65 consists of 2 functional regions. The carboxy-terminal 78 amino acids function as an activating region (Sadowski et al., 1988; Triezenberg et al., 1988; Greaves and O'Hare, 1989) while the amino terminal 413 amino acids are responsible for the ability of the protein to form complexes with the TAATGARAT motif in a sequence specific fashion (Triezenberg et al., 1988; Ace et al., 1988; Greaves and O'Hare, 1989; this thesis). No direct evidence bears on the question of whether the acidic domain is involved in the activity of Vmw65 as a component of the virion structure, however the absence of this region from VZV orf10 suggests that it is not.

An intriguing aspect of Vmw65, given its role as a sequence specific transactivator, is that it fails to show any substantial affinity for double stranded DNA (Marsden et al., 1987). This apparent contradiction was resolved by the demonstration that both cellular proteins and the TAATGARAT motif are required to allow Vmw65 to interact specifically with DNA (McKnight et al., 1987; Preston et al., 1988;

O'Hare and Goding, 1988). The identity of one of the cellular components involved in the complex was suggested by the ability of the octamer sequence (ATGCAAAT) to compete for proteins binding to the TAATGARAT motif (O'Hare and Goding, 1988). These data were confirmed by the demonstration that a purified preparation of a HeLa cell factor, named octamer transcription factor (OTF) 1, was required for the formation of a Vmw65-containing tertiary complex at the TAATGARAT motif (Gerster and Roeder, 1988). These workers also suggested that a second cellular factor was involved in complex formation as reactions containing only purified OTF-1 and Vmw65 synthesised *in vitro* failed to bind efficiently to oligonucleotides containing the TAATGARAT motif. Binding activity could be restored with OTF-1 depleted HeLa cell extract.

The requirement for OTF-1 in Vmw65 activity led to the reassessment of TAATGARAT sequences upstream of HSV IE genes and the demonstration that many of them represent a combined octamer/TAATGARAT binding site (O'Hare and Goding, 1988, ApRhys *et al.*, 1989), though the TAATGARAT motif alone is sufficient to bind cellular factors, form tertiary complexes and mediate Vmw65-induced transactivation (Bzik and Preston, 1986). Conversely, the octamer sequence alone, while binding cellular factors efficiently, is not capable of supporting tertiary complex formation or Vmw65 mediated transactivation (Post *et al.*, 1981; Gerster and Roeder, 1988; O'Hare *et al.*, 1988; this thesis). The only data incompatible with this model is that presented by O'Hare and Goding, (1988) who report Vmw65 stimulation of CAT expression from a plasmid encoding the CAT gene under the control of the HSV TK promoter and a single octamer sequence, however no evidence was provided that this sequence could mediate tertiary complex formation, and thus its significance remains unclear.

Mutational analyses of Vmw65 have confirmed the model of two functional domains. Triezenberg et al., (1988a) produced a series of carboxy-terminal deletions and showed that the 78 amino acid residues at the carboxy-terminus were crucial for the activity of the protein in stimulating transcription. Truncated proteins derived from Vmw65 but encoding only part of the acidic region showed intermediate

activities when assayed in short term transfection assays. This suggested that the number of negatively charged residues and not necessarily the integrity of the acidic region, was important in the ability of the protein to stimulate transcription, a finding in accordance with results from other systems (Hope et al., 1988). In addition Triezenberg et al., (1988) presented indirect evidence as to the role of the amino terminal portion of the protein. In the absence of the acidic tail the amino terminal segment acted dominantly and specifically to interfere with the trans-inducing activity of wt Vmw65 polypeptides, suggesting competition for binding to the cellular proteins involved in the tertiary complex. Ace et al., (1988) presented more detailed data as to the location of functionally important regions within the polypeptide. They constructed a series of in frame, four amino acid, insertion mutants throughout the orf and assayed these for their ability to stimulate transcription, form tertiary complexes in an in vitro binding assay and fulfil the protein's structural role by rescuing the HSV-2 mutant ts2203. Of the ten mutants described five failed to rescue ts2203, and four had at least a partial defect in both transactivation and tertiary complex formation. One insertion, mutant in14, was found to produce a protein which was able to rescue ts2203 but not to transactivate IE gene expression or to form tertiary complexes. These results had three important implications, first, transcriptional activity correlated closely with tertiary complex formation, second, amino acids required for the formation of tertiary complexes are widely distributed within the amino terminal domain of the protein and third, the protein's structural and transactivating roles are separable.

A major step towards understanding the biological relevance of the stimulation of IE genes by Vmw65 was made with the construction of an HSV-1 mutant, in1814 (Ace et al., 1989). This virus contains the lesion present in the mutant Vmw65 protein in14 described above (Ace et al., 1988) and thus produces a Vmw65 molecule which fails to stimulate IE gene expression but fulfils its role in virion structure. This deficit resulted in a surprisingly small reduction in the accumulation of IE mRNAs when in1814 was grown in tissue

culture under cycloheximide block. Levels of IE RNA 1 and 2 were reduced five fold, while those of IE RNA 4 were reduced only two fold and IE 3 accumulation remained unchanged. Mutant in1814 had a high particle to pfu ratio which was even more pronounced on slow growing cell types such as HFL. Interestingly this aspect of its phenotype is similar to that of mutants dl1403 and FXE which lack functional Vmw110 protein (Stow and Stow, 1986; Everett et al., 1989; section 1.3.2.4.) suggesting the possibility of similar roles at different stages of the virus life cycle for these gene products.

Infection of mice with in1814 revealed that the mutant is essentially avirulent (Ace et al., 1989), not surprising given the growth restricted nature of the virus in tissue culture. Analysis of the mutant in the mouse eye model of latency revealed that the virus goes through the full cycle of induction, maintenance and reactivation without the usual steps of replication at the site of inoculation and in the ganglion (Steiner et al., submitted).

These data are consistent with a view of Vmw65 as one of several factors, possibly including Vmw110, which act to commit the viral genome to a lytic form of expression. Transactivation by Vmw65 is not essential in tissue culture where its role can be duplicated by large amounts of input virus, or by cellular transcription factors, presumably present at higher concentrations in faster growing cells. However, in the more stringent environment of an infected animal or in a situation where the numbers of genomes are restricted in tissue culture, the deficit in transactivation results in fewer genomes being committed to the lytic cycle and thus defects in replication and virulence in vivo and a proportionately higher particle to pfu ratio in vitro.

The in vitro phenotype of in1814 was partially reproduced in another set of studies using a cell line constitutively expressing a mutant Vmw65 protein lacking the 78 amino acid carboxy-terminal acidic region (Friedman et al., 1989). This cell line showed reduced permissiveness for viral replication, especially when the virus was inoculated at low multiplicities, which correlated with decreased expression of its IE genes. The authors suggested that these findings could be explained by a model where the

amino terminal portion of the protein competed with wild type Vmw65 in the formation of complexes on TAATGARAT sequences, thus reducing transactivation.

1.4.3. Octamer Binding Proteins.

The octamer motif (ATGCAAAT) was initially characterised as a *cis*-acting sequence which conferred B-cell specific transcription on the immunoglobulin light and heavy chain genes. It is present in both the promoter and enhancer elements of these genes (Falkner and Zachau, 1984; Parslow *et al.*, 1984; Mason *et al.*, 1985).

This motif is also found upstream of genes not specific to lymphoid cells, notably the histone H2B gene (LaBella *et al.*, 1988; Sturm *et al.*,1988), the U2 snRNA gene (Ares *et al.*, 1985; Mattaj *et al.*, 1985; Mangin *et al.*, 1986), the control regions of SV40 E genes (Zenke *et al.*, 1988), the HSV thymidine kinase and HSV IE genes (McKnight and Kingsbury, 1982; ApRhys *et al.*, 1989), and also in the adenovirus origin of DNA replication (Pruijn *et al.*, 1986; Rosales *et al.*, 1987). However, the detection of the octamer sequence, and in many cases the demonstration of its ability to interact with proteins, has not always correlated with evidence of the functional significance of this sequence in the stimulation of transcription in non-lymphoid cells (Preston *et al.*, 1984; Davidson *et al.*, 1986; Tanaka *et al.*,1988).

To date three proteins (Oct-1, Oct-2a and Oct-2b) which interact with the octamer motif have been purified and for two of these (Oct-1 and Oct-2a) sequence data are available (Sturm *et al.*, 1988; Clerc *et al.*, 1988; Scheidereit *et al.*, 1988; Schreiber *et al.*, 1988). Oct-1 is a protein of 100 kda, also referred to as OTF-1 (Fletcher *et al.*, 1987), OBP100 (Sturm *et al.*, 1987), NFIII (O'Neill *et al.*, 1988) and NF-A1 (Staudt *et al.*, 1986) and found in a wide range of cell types, while Oct-2a and Oct-2b, proteins of 60 kda and 75 kda respectively, are confined to lymphoid cells (Landolfi *et al.*, 1986; Singh *et al.*, 1986; Schreiber *et al.*, 1988). Analysis of the amino acid sequence of these proteins revealed the presence of a homeo domain, a

conserved amino acid sequence found in a class of proteins important in the control of developmental processes (see section 1.5.3.1.). Close to this domain a second conserved sequence was found which defined a subset of homeobox proteins now known as POU proteins (Herr et al., 1988). Members of the POU protein family initially comprised Oct-1, Oct-2, Pit-1 which is synthesised in pituitary cells and implicated in the activation of growth hormone and prolactin genes (Bodner et al., 1988; Ingraham et al., 1988) and Unc86, which has a genetically defined role in the differentiation of specific nematode neural cells (Finney et al., 1988), thus POU from Pit-1, Oct and Unc86. More recent data have added to this list (He et al., 1989; Scholer et al., 1989), though functional analyses of these new protein species is still awaited. The Oct-1 and Oct-2 proteins show considerable homology within their POU and homeo domains (98% and 88% respectively) which have been shown to be responsible for their interaction with DNA (Sturm et al., 1988; Clerc et al., 1988; Scheidereit et al., 1988). However outside these regions the proteins have little similarity in sequence, suggesting considerable differences in their mechanism of action (Herr et al., 1988).

An interesting aspect of the function of both Oct-1 and Oct-2, that has emerged through the study of purified preparations of these proteins, is the wide range of DNA sequences with which they can interact. Analysis of interactions with variants of octamer sequences (Baumruker et al. 1988; Kelmer et al. 1989) revealed that the protein will recognise degenerate sequences as diverse as TAATGARAT and CTCATGA, though with less affinity than the prototypic octamer motif. Sequences flanking the 8 bp core become increasingly important in facilitating interactions with sequences less closely matched to the octamer motif (Baumruker et al., 1988).

A great deal of evidence has accumulated regarding the DNA-protein interactions of the octamer sequence in both lymphoid and non-lymphoid cells, however the functional significance of this motif has been the subject of some debate. In lymphoid cells a direct correlation has been established between the presence of octamer elements in promoters and enhancers and the stimulation of transcription

(Davidson et al., 1986; Gerster et al., 1987; Lenardo et al., 1987; Wirth et al., 1987). However repetition of these experiments in non-lymphoid cells has, in general, resulted in unaltered levels of transcription (Davidson et al., 1986; Zenke et al., 1986; Ondek et al., 1987; Tanaka et al., 1988). Thus the same motif, or set of motifs, appears to stimulate transcription in lymphoid cells but not in other cell types tested, despite interacting with proteins in both systems.

Three exceptions to this deficiency of octamer mediated transcription in non-lymphoid cells have been described. First, expression of the U2 snRNA gene is dependent on the presence of a functional octamer binding motif in its 5' control region (Mattaj et al., 1985; Manjin et al., 1986). Second, the stimulation of expression of the human histone H2b gene, during the S phase of the cell cycle results from the presence of an octamer motif (LaBella et al., 1988), and third the stimulation of HSV IE gene expression, by the virion protein Vmw65 (Gerster et al., 1988), is dependent on the binding of Oct-1 to the degenerate octamer sequence TAATGARAT. However, in both of the latter two cases baseline gene expression (that is non-S phase and without Vmw65) is unaffected by the presence or absence of octamer motifs or their derivatives (LaBella et al., 1988; Preston et al., 1984; Bzik and Preston, 1986).

Recent data from Herr's laboratory (Stern et al., 1989) have identified the homeo domain of Oct-1 as the site necessary for the interaction with Vmw65. They achieved this by showing that a fragment containing only the POU and homeo box sequences was able to form a complex with TAATGARAT and Vmw65. A series of domain exchanges between Oct-1, Oct-2 and Pit-1 then localised the site of interaction to the homeo box. Seven amino acid changes in this 60 amino acid domain distinguish Oct-1 from Oct-2, conversion of three of these from the Oct-1 sequence to the Oct-2 sequence, abrogated the interaction of Vmw65 with Oct-1. These changes affect residues on the solvent exposed face of a predicted α-helix. Reciprocal changes did not confer the ability to form a complex on Oct-2. Interestingly, the important region of the homeo box shows potential structural similarity to the DNA binding domain of

the repressor. Equivalent mutations in this protein have been shown to affect the regulatory interactions of this protein without interfering with its DNA binding activity. In conclusion Stern et al., (1989) suggest that the conservation of the homeo box through evolution results at least in part from its ability to form protein-protein as well as protein-DNA interactions.

The demonstration that the DNA binding domain of Oct-1 is intimately involved in its interaction with Vmw65 also relates to the alteration in binding specificity from ATGCAAAT to TAATGARAT that occurs as a result of the interaction (Preston et al., 1988; this thesis). It is possible that the lower affinity interaction between Oct-1 and TAATGARAT is necessary to allow interaction with Vmw65.

1.4.4. IE Gene Regulation in Other Alpha-Herpesviruses

In comparison to the large body of knowledge concerning the regulation of the IE genes of HSV, information regarding the regulation of other α-herpesvirus IE genes, is minimal. One study has reported the sequence and characterisation of the regulatory region of the pseudorabies virus (PRV) major IE gene (Campbell and Preston, 1987). These data revealed the presence of potent regulatory sequences, including repeated motifs which showed some similarity to those present in the enhancers of HCMV and murine cytomegalovirus (MCMV) major IE genes. The PRV IE gene regulatory sequences were responsive to Vmw65 though no TAATGARAT motif was detected and PRV does not appear to encode a homologue of Vmw65 (Hampl et al., 1984). Several copies of the octamer motif are present in the regulatory region though it would contrast with the HSV system if these were the sequences responsible for the Vmw65 responsiveness. This study also demonstrated that a Vmw65 homologue is not necessary for the expression of α-herpesvirus IE genes, a finding confirmed by Ace et al., (1989), and suggest that either a cis-acting sequence bound by a cellular factor or a simple promoter strength model are sufficient to distinguish IE genes from the other temporal classes.

Only one allusion to the sequences regulating a VZV IE gene has been made. This involved the stimulation of

expression in cell lines stably transformed with VZV orf62 with its own regulatory sequences by infection with HSV-1 under IE conditions suggesting that this promoter is also responsive to Vmw65 (Felsner et al., 1988). The lack of a published mRNA start site for the transcript precluded further analysis of the regulatory sequences.

1.5. Eukaryotic Transcriptional Regulation

1.5.1. Cis-Acting Sequences

Control of transcription by RNA polymerase II in eukaryotic cells is mediated, at the most basic level, through the specific interaction of proteins and DNA sequences. Initial experiments attempted to define these sequences by deletion and mutagenesis of control regions and analysis of the effects of these manipulations on the level of transcription in cells or cell free systems. Data from these studies have revealed basic patterns in the arrangement and function of control sequences (Maniatis et al., 1987).

Taking the 5' end of the mRNA as +1, a short A+T rich sequence, known as the TATA box, is found at or around -28. Mutations in this motif have been shown to affect the accuracy of mRNA initiation to a larger extent than the rate of transcription (Grosscheld and Birnsteil, 1980; Benoist and Chambon, 1981; Ghosh et al., 1981). The TATA box is the most highly conserved cis-acting motif in genes transcribed by RNA polymerase II and is thought to be involved in the formation and positioning of a multisubunit complex incorporating the enzyme itself. However TATA boxes are absent from a significant subset of genes transcribed by this enzyme (Melton et al., 1984; Reynolds et al., 1984; Valerio et al., 1985). Some of these have been shown to fulfill cellular "housekeeping" functions and to have their 5' ends in so called CpG rich islands, though the connection between these observations is not clear (Bird, 1986).

The region upstream of the TATA box contains upstream activating sequences (UASs). These are short (8-12bp) motifs which act to stimulate transcription either constitutively, as in the case of CCAAT box elements (Myres et al., 1986) and SpI binding sites (Jones and Tjian, 1985), or in response to specific stimuli such as serum stimulation (Treisman, 1985), heat shock (Bienz and Pelham, 1986), viral infection (Goodbourn et al., 1985, 1986), exposure to heavy metals (Serfling et al., 1985) and steroids (Renkawitz et al., 1984). These cis-acting sites have all been shown to mediate their effects through interactions with proteins.

The motifs can be present in either orientation and show a decrease in activity with increasing distance from the TATA box (McKnight et al., 1982). More subtle changes in positioning can also affect transcription, the insertion of oligonucleotides that alter spacing by a half turn of the DNA helix results in a more profound reduction in transcriptional activity than those that result in a full turn (Takahashi et al., 1986).

A second group of sequences that act to stimulate transcription are known as enhancers. These have been defined by their ability to act over considerable distances both upstream and downstream of the mRNA start site and by their independence of orientation in relation to the gene. They are longer than UASs, sometimes comprising hundreds of bases (Edlund et al., 1985), and contain binding sites for many different proteins (Serfling et al., 1985).

The enhancer sequence studied in most detail forms part of the SV40 E gene regulatory sequences. These consist of a TATA box and two upstream control elements. The proximal of these comprise three 21 bp repeats each of which in turn contains two binding sites for the transcription factor Spl. These act as UASs (Myres et al., 1981). The distal control region consists of two 72 bp repeats (Banerji et al., 1981; Gross et al., 1981; Moreau et al., 1981). Each of the 72 bp repeats can be divided into three 15-20 bp elements called A, B and C that cooperate to form the enhancer (Zenke et al., 1986; Herr and Clarke, 1986; Herr and Gluzman, 1985; Clarke and Herr 1987). These enhancer elements function autonomously when present in two or more copies and display unique patterns of cell specific enhancer activity (Ondek et al., 1987; Schirm et al., 1987). Enhancer function is maintained even when elements are separated by up to 100 bp (Ondek et al., 1988). Enhancer elements are in turn composed of sequence motifs which are individual protein binding sites. Enhancer motifs differ from elements in that there appear to be strict constraints on the spacing between the individual motifs within elements. The activity of multimers of enhancer elements is completely lost if individual motifs are separated by as few as five bases (Ondek et al., 1988).

A further question, as yet unresolved, is whether

enhancers and UASs are qualitatively different in terms of their mechanism of action, or whether the unique features of enhancers relate simply to the presence of a large number of protein binding sites. Several lines of evidence argue against a qualitative difference between UASs and enhancers. First, the same sequence motifs such as activating protein 1 (AP1) binding sites (Lee et al., 1987) and the octamer motif (Bohmann et al., 1987b; Parslow et al., 1987) act in both UASs and enhancers. Second, the SV40 enhancer can replace UASs deleted from the β-globin promoter (Treisman and Maniatis, 1985). Third, while a single heat shock upstream promoter element fails to act at a distance, duplication of this motif gives rise to an element with all the properties of an enhancer (Bienz and Pelham, 1986). This last phenomenon could not be reproduced when CCAAT box motifs were duplicated (Bienz and Pelham, 1986).

In favour of qualitative differences between promoter and enhancer sequences is the finding that, while similar sequence motifs exist in promoters and enhancers, they may interact with different proteins (Muller et al., 1988). In addition, a body of evidence suggests that transcription stimulated by enhancers can be suppressed by proteins such as the adenovirus E1A product (Velcich and Ziff, 1985; Hen et al., 1985; Stein and Ziff, 1987) and a cellular equivalent present in undifferentiated but not differentiated F9 cells (Germain et al., 1985). These proteins reduce enhancer driven transcription while leaving promoter driven transcription unaffected, but their mechanism of action is not clear.

Thus, the source of functional differences between UASs and enhancers is not known, however, the close juxtaposition of numerous protein binding sites in an enhancer does appear to result in activity that is qualitatively different from the sum of its parts.

1.5.2. Proteins Involved in Transcriptional Regulation

Cis-acting elements mediate control over the level and tissue distribution of transcription largely through specific interactions with proteins. Detailed knowledge of the

proteins and their interactions with DNA will be necessary to understand the mechanisms involved in the regulation of transcription.

Given the diversity of cis-acting motifs and evidence that they act by forming specific interactions with protein factors, a one-to-one relationship between factor and motif seemed likely. This model proved too simplistic. Recent studies have provided examples of different motifs which interact with the same protein (Baumruker et al., 1988), different proteins that bind to the same motif (Chodosh et al., 1988) and protein-DNA interactions that involve single proteins, homodimers (Struhl, 1987) or an array of different proteins (Preston et al., 1988; Gerster and Roeder, 1988). Given this complexity, no attempt will be made to describe every factor that has been reported, detailed descriptions of only those three families of factors directly involved in this project will be given. These, the octamer binding proteins (section 1.4.3.), the activating transcription factor (ATF)/cyclic AMP response element (CRE) binding protein and AP1 family and the CCAAT box binding proteins, do, however, provide good examples of some of the problems and surprises in this field.

1.5.2.1. ATF/CREB Protein Family

The transcriptional regulatory proteins which bind to ATF/CRE motifs are proving to be an important model for the mechanisms which control gene regulation. The binding site core was derived from two separate sources. Analysis of a series of cellular genes whose transcription was known to be responsive to cAMP led to the identification of the core sequence TGACGTCA that bound a factor involved in cAMP dependent gene regulation (Short et al., 1986; Montminy et al., 1986; Comb et al., 1986; Silver et al., 1987; Tsakada et al., 1987). Concurrently, work on the regulation of adenovirus early gene regulation led to the identification of the same sequence as binding a cellular factor (named ATF) involved in the stimulation of transcription. This cis-acting sequence was found in the promoter regulatory regions of the adenovirus E1A, E2, E3 and E4 genes (Hurst and Jones, 1987; Lee and Green, 1987). Further analysis

demonstrated that the ATF binding motif conferred cAMP responsiveness to these genes both in short term transfection assays and during viral infections. In addition, responsiveness to the adenovirus transactivating protein E1A, could also be mapped to this motif (Lee and Green, 1987).

Several lines of evidence suggest that the rigid conservation of the consensus TGACGTCA is neither necessary nor sufficient for cAMP responsiveness. In some situations non-consensus motifs have been shown to stimulate transcription more effectively than the consensus sequence and in others sequences flanking the core motif are essential for cAMP responsiveness (Montminy et al., 1987; Bokar et al., 1988; Deutch et al., 1988). Furthermore, it is likely that a family of proteins bind to the ATF consensus (see below), and flanking sequences may play a vital role in selecting the members of the family that bind and thus the functional characteristics of the binding site.

Running concurrently with the identification of cAMP responsive genes and the analysis of their cis-acting motifs has been the analysis of the proteins which interact with them. The first report of purification of a CRE binding protein was by Montminy and Bilezikjian (1987) who identified a nuclear phosphoprotein of molecular weight 43,000 which they purified from both the phaeochromocytoma cell line (PC12) and rat brain, using affinity to the CRE of the rat somatostatin gene. This protein was found to stimulate transcription of the cAMP responsive gene in vitro and bound to the CRE as a dimer (Montminy and Bilezikjian, 1987)

More recently, cloning and sequencing of the genes encoding ATF/CRE binding proteins has been achieved. Two groups have published very similar sequences of CRE binding proteins from a human placental λgt11 library (Hoeffler et al., 1988) and from rat brain (Gonzalez et al., 1989). These proteins had apparent molecular weights of 38kd and 43kd on SDS-PAGE, however their predicted molecular weights from sequence data were 35kd and 38kd respectively (Hoeffler et al., 1988; Gonzalez et al., 1989). A third group has published the sequence of a 54.5kd CRE binding protein from a human brain cDNA library (Maekawa et al., 1989). While

the amino acid sequence of this protein is largely unrelated to the others published, all three proteins share important features related to their function and control. A carboxy-terminal "leucine zipper" and DNA binding domain (section 1.5.3.3.) is present in all of the proteins suggesting a relationship to the large family of DNA binding proteins that contain this motif. In addition the proteins encode sites for phosphorylation by protein kinase C and protein kinase A, and the 35/38kd proteins contain a potential phosphorylation site for caesin kinase II (Gonzales et al., 1989). Further sequence analysis revealed that the amino-terminal 268 amino acids of the 38kd protein has an acid to base ratio of 2.3 to 1, suggesting that an acidic activating domain might be present (Hoeffler et al., 1987; section 1.5.4.). In addition, residues 10-100 and 160-240 of this protein contain approximately 20% glutamine residues raising the possibility that this protein may act to stimulate transcription by two different mechanisms (section 1.5.4.). The 54.5kd protein does not possess a notably acidic region (Maekawa et al., 1989), however 18 proline residues are present in a region between residues 200 and 272 suggesting that, like a similar sequence in CTF/NF1 (section 1.5.4.), this too may represent an activating region.

The mechanism by which increased levels of cAMP alter these transcription factors to induce increased gene expression is currently the subject of intense study. The CRE /ATF family are known to be phosphoproteins which bind to their cognate sites as dimers and it has been shown that transcriptional activity both in cells and cell free extracts can be stimulated by the catalytic subunit of the cAMP dependent protein kinase and inhibited by its regulatory subunit (Grove et al., 1987; Day et al., 1989). As mentioned above, CRE binding proteins thus far analysed contain phosophorylation sites for several protein kinases. The functional relevance of these sites was demonstrated by Yamamoto et al., (1988) who found that phosphorylation by protein kinase C resulted in increased dimer formation, while phosphorylation by protein kinase A resulted in a 20 fold increase in transcription in the in vitro assay used. This study also showed that the removal of phosphate

residues causes a reduction in dimer formation, binding and transcriptional activation.

In vivo, the adenovirus E1A protein acts to stimulate adenovirus E gene expression (see sect. 1.5.2.4.). ATF binding sites have been directly implicated in this activity though the mechanism remains controversial. Several attempts have been made to determine whether E1A and cAMP-mediated transcriptional stimulation are related. Evidence available to date has not clarified the situation since single, synergistic and separate pathways have been reported (Engel et al., 1988; Sassone-Corsi, 1988; Leza and Hearing, 1989). These contrasting findings may be due to differences in cell types and species of origin, to the assays employed (virus infection compared with DNA transfection) or to the different promoter regions analysed. Further work will be required to differentiate between the possibilities.

1.5.2.2. Activating Protein 1

The AP1 binding site (TGAGTCA) bears a striking resemblance to the CRE core (TGACGTCA), and indeed these two sites show 10%-15% cross-competition in protein binding studies (Hurst and Jones, 1987). Despite this similarity, the functions mediated by AP1 binding proteins differ markedly from those bound by CREs.

The AP1 binding consensus was derived from analysis of genes whose transcription was found to be induced by the tumour promoter TPA (Lee et al., 1987; Angel et al., 1987; Piette and Yaniv, 1987). This core sequence, like the CRE, was shown to interact specifically with a series of protein species and to confer TPA responsiveness on heterologous promoters from a position either 3' or 5' to the reporter gene sequences (Angel et al., 1987). Progress towards identifying the proteins involved in AP1 activity was made with the discovery that the AP1 binding site is very similar to that of the yeast transactivator GCN4 (consensus ATGA(^{C}/G)TCAT) which is involved in the regulation of aspects of amino acid metabolism (Hope and Struhl, 1986). The similarity of these two sites suggested that there might also be a connection between the proteins binding to them

(Struhl, 1987).

Independently Vogt et al., (1987) demonstrated sequence similarity between GCN4 and an oncogene, jun, which is responsible for the induction of fibrosarcomas in chickens by avian sarcoma virus 17 (Maki et al., 1987). The similarity between AP1 and GCN4 binding sites and between the proteins jun and GCN4 led to the demonstration that jun was responsible for part of the DNA binding activity defined as AP1 (Bohman et al., 1987; Angel et al., 1988; Bohman et al., 1988). More recent data have revealed that jun is one of a family of related proteins. Two others, jun B and jun D, have also been sequenced (Ryder et al., 1988, 1989). Comparisons of DNA sequences of the family of jun proteins revealed that regions thought to be important in dimerisation, interactions with DNA and stimulation of transcription show over 70% similarity amongst the three proteins. The remainder of the predicted amino acid sequences show a much lower level of similarity, recalling the situation with the ATF protein family (Section 1.5.2.1.).

Studies on another oncogene product, fos, which is responsible for the induction of osteogenic sarcomas by the FBJ murine sarcoma virus (Curran and Teich, 1982) showed that it too contributes to AP1 binding activity. The cellular homologue of v-fos was known to be a nuclear phosphoprotein found in complexes with a second, 39kd, protein (Curran et al., 1984) and several pieces of evidence suggested that it was involved in gene regulation (Cohen and Curran, 1988; Sambucetti and Curran, 1988; Distel et al., 1987). A series of studies then led to the revelation that the 39kd protein that bound with fos in the DNA binding complex was jun (Rauscher et al., 1988; Chiu et al., 1988; Sassone-Corsi et al., 1988; Lamph et al., 1988). Sequence comparisons between fos and related proteins, and peptides derived from AP1, demonstrated that these proteins also contributed to AP1 binding activity. Additional studies revealed that antibodies against fos could inhibit binding of proteins to the AP1 motif (Rauscher et al., 1988). In addition, comparisons of the sequences of fos and related proteins with those of the jun protein family and other DNA binding proteins showed that the fos and jun protein

families contained leucine zipper motifs and associated DNA binding domains (Landschultz et al., 1988b; section 1.5.3.3.).

Thus AP1 binding activity appears to be dependent on co-operative interactions between the fos and jun protein families. It is likely that the two constituents of the complex contribute equally to DNA binding activity (section 1.5.3.3.) but their relative contributions to transcriptional activation are not known. It is also known that members of the fos and jun protein families can form heterodimers with at least some of the members of the complementary family in vitro (Turner and Tjian, 1989). This obviously provides scope for great flexibility of interactions in vivo. The extent and functional significance of these interactions await further analysis.

To generalise further, the fos/jun and ATF/CREB protein families appear to represent important elements in the control of cellular transcription. There are striking similarities between these protein families. All bind, as dimers, to similar DNA sequences, they share significant homology in sequences that control protein dimerisation and protein-DNA interactions and their transcriptional activities are altered by post-translational mechansims. The extent and significance of interactions amongst the fos, jun and ATF/CREB families in vivo is not clear but is obviously of great interest. It should also be noted that while most of the similarities between proteins in all three families centres on their DNA binding domains, the remainder of the proteins must contribute sequences of relevance to the proteins' individual roles. The analysis of these sequences will be of great importance.

1.5.2.3. CCAAT Box Binding Factors

CCAAT box motifs are present in the UASs of many genes transcribed by RNA polymerase II (Mellon et al., 1981; Jones et al., 1985; Myres et al., 1986; Graves et al., 1986; Morgan et al., 1988), and are important in the stimulation of transcription (Graves et al., 1986; Myres et al., 1986). A related site was also found to be responsible for stimulating the replication of adenovirus DNA (Nagota et

al., 1982; Rawlins et al., 1984; Hay, 1985).

Purification of proteins that interact with both transcription and replication sites yielded surprising results. Proteins purified by affinity chromatography using CCAAT motifs from the human β-globin and human ras genes yielded several species with molecular weights between 52 and 66kd which were named CCAAT transcription factor (CTF) 1 (Jones et al., 1987). These were shown to be structurally and functionally identical to protein species, named nuclear factor (NF) 1, purified using a consensus derived from the adenovirus origin of replication (Rosenfeld and Kelly, 1986). Concurrently, a second CCAAT box binding protein was purified using the HSV TK promoter and murine sarcoma virus long terminal repeat and called CCAAT binding protein (CBP) (Graves et al., 1986). CBP could be distinguished from CTF/NF1 by heat stability and binding affinities for mutant CCAAT box motifs (Graves et al., 1986). This protein was also found to be identical to a second species purified by Johnson et al., (1987) using a completely unrelated sequence $TGTGG^{A}/T^{A}/T^{A}/TAG$, an "enhancer core homology" common to many animal virus enhancers (Weiher et al., 1983). It was initially named enhancer binding protein (EBP) but when it was shown to be identical to CBP the names and acronyms were combined resulting in C/EBP (Johnson et al., 1987). Studies using proteolysed fragments of the protein showed that both CCAAT and enhancer core binding activities co-localised to a 14kd domain. Sequence data are available for C/EBP and CTF/NF1 (Landschultz et al., 1988a; Santoro et al., 1988). Comparison of these confirmed that the proteins are encoded by different genes and, surprisingly, that no similarity exists between the DNA binding domains of the proteins. C/EBP contains a leucine zipper motif and associated DNA binding domain (Landschultz et al., 1988; section 1.5.3.3.), while the CTF/NF1 sequences responsible for binding DNA do not resemble any of the currently recognised motifs (Santoro et al., 1988). Sequencing of CTF/NF1 cDNAs also revealed that transcripts of this gene could be spliced in at least three ways to form a family of factors (Santoro et al., 1988). This at least partially explains the heterogeneity in molecular weights seen in purified preparations of the protein (Jones et al., 1987). Thus the same cis-acting

motif can interact with at least two entirely distinct proteins whose DNA binding domains have no detectable sequence similarity.

The heterogeneity and complexity of the CCAAT box binding proteins does not stop here. In addition to C/EBP and CTF/NF1, numerous apparently different CCAAT box binding activities have been purified both from different cell types and also from the same cells (Hatamotchi et al., 1986; Cohen et al., 1986; Barberis et al., 1987; Dorn et al., 1987; Chodosh et al., 1988; Raymondjean et al., 1988; Gallinari et al., 1989).

The proteins purified by Chodosh et al., (1988) are of particular interest. They consist of two novel species purified from HeLa cells, CCAAT box binding proteins (CP) 1 and 2, which were distinguishable by their different affinities for individual CCAAT box motifs. Both proteins bound DNA most efficiently in the form of heterodimers. A fascinating insight into the complexity of gene regulatory mechanisms and their conservation across species boundaries came from the demonstration that the two components of CP-1 (CP-1A and CP-1B) could form functional heterodimers with the yeast proteins HAP-2 and HAP-3 which control catabolite derepression of cycl gene expression in Saccharomyces cerevisiae (Guarente et al., 1984; Pinkman and Guarente, 1985). Complexes formed between CP-1A and HAP-2 and CP-1B and HAP-3 (Chodosh et al., 1988b). A further component of the HAP2/HAP3 complex has recently been identified (Forsburg and Guarente, 1989). This protein, termed HAP4, is necessary for DNA binding by the HAP2/HAP3 complex in vitro and contains a highly acidic region, necessary for the activity of the complex. Mutant strains from which this region was absent had lost the ability to regulate cycl expression (Forsburg and Guarente, 1989). These mutants could be rescued by replacing the acidic activating region of HAP4 with that of GAL4 (Forsburg and Guarente, 1989).

Thus CCAAT box binding proteins represent a structurally and functionally diverse group of proteins, whose only connection appears to be their affinity for the pentanucleotide CCAAT. Their importance is emphasised by the widespread use of this motif in promoter sequences and, in at least one case, the functional conservation of

protein-protein interactions between yeast and man.

1.5.2.4. Adenovirus E1A Protein

A second overall group of transcriptional regulatory proteins exists, which may function in a fundamentally different fashion from those described above. It includes viral immediate early proteins such as the adenovirus early region 1A (E1A) gene products, the pseudorabies virus major IE gene product and the HSV IE gene product Vmw110. These do not appear to rely on sequence specific DNA interactions to mediate their effects and are able to transactivate a variety of promoters by mechanisms that are not yet fully understood (Berk, 1986; Everett, 1989). Their functional similarity has been demonstrated by experiments in which co-infection with pseudorabies virus was shown to allow an adenovirus mutant (dl312), which lacks a functional E1A gene, to grow in tissue culture (Feldman *et al.*, 1982; Imperiale *et al.*, 1983). In addition, cotransfection of E1A stimulates expression of HSV E gene expression in short term transfection assays (Everett and Dunlop, 1984). However, no direct evidence exists to prove that the mechanisms of action of these different proteins are similar.

The products of the adenovirus E1A gene are multifunctional, nuclear phosphoproteins which transactivate other adenovirus E genes, immortalise primary rodent cells, repress the activity of transcriptional enhancers and influence cellular differentiation (Berk, 1986; Moran and Matthews, 1987).

The E1A gene of group C adenoviruses produces two major spliced products early in infection, the 12S and 13S mRNAs, which encode proteins of 243 and 289 amino acids, respectively. These proteins are distinguished only by 46 amino acids at positions 140-185 in the 13S product, which are highly conserved amongst several adenovirus serotypes (Kimelman *et al.*, 1985) and are essential for stimulation of E gene expression. Other areas of conservation between adenovirus serotypes exist between residues 40 and 80 (Domain 1) and 121 and 140 (Domain 2). These have been shown to be essential for the other roles of E1A (Moran and

Matthews, 1987).

The presence of E1A alone is insufficient to induce cells to a fully transformed phenotype, though both the 12S and the 13S products are equally efficient in immortalising cells (Haley et al., 1984; Roberts et al., 1985; Zerler et al., 1986). Complete transformation requires expression of the E1B region (Houweling et al., 1980; van den Elson, 1983) though this function can be replaced by an activated H-ras oncogene or the polyoma virus middle T antigen (Ruley, 1983). These immortalisation functions of E1A are unaffected by deletions of sequences beyond residue 139 and thus are unlikely to depend on transactivation (Zerler et al., 1987).

Adenovirus infection is also known to stimulate DNA replication in serum starved, G_o arrested, primary or secondary cells (Shimojo and Yamashita, 1968). This function has also been mapped to E1A and is presumably related to the proteins' ability to induce the immortalisation of cells (Braithwaite et al., 1983).

Initial data suggested that the repression of enhancer driven transcription was linked to the function of immortalisation and induction of DNA synthesis, however, more recent studies have isolated mutants in domain 2 that affect transcriptional repression alone (Velcich and Ziff, 1988; Subramanian et al., 1988).

The mechanism of transcriptional stimulation by E1A has been the subject of considerable interest. Adenovirus mutants with lesions in E1A produce decreased levels of early region transcripts (Nevins, 1981) and replicate very poorly in HeLa cells (Harrison et al., 1977; Jones and Shenk, 1979). However, like the HSV-1 mutants in1814 (Ace et al., 1989), dl1403 (Stow and Stow, 1986) and FXE (Everett, 1989), this defect is much less severe at high multiplicities of infection (Shenk et al., 1979). E1A also stimulates the expression of numerous cellular genes, transcribed by either RNA polymerase II or III, in short term transfection assays (Green et al., 1983; Svensson and Akarjarvi, 1984; Allan et al., 1984 Gaynor et al., 1984, 1985; Stein and Ziff, 1984; Hoeffler and Roeder, 1985), though it fails to stimulate the transcription of their endogenous counterparts (Green et al., 1983; Babich et al.,

1983; Gaynor et al., 1985) with the exception of the gene encoding heat shock protein 70 (Kao and Nevins, 1983) and a B-tubulin gene (Stein and Ziff, 1984).

Evidence that E1A uses a different mechanism of activation from other transcriptional activators has come from several studies. Firstly, E1A fails to make sequence specific interactions with DNA (Chatterjee et al., 1988). Secondly, target sequences responsible for E1A mediated transactivation are also responsible for transcriptional activity in the absence of E1A. These motifs have been shown to bind cellular proteins involved in the regulation of transcription such as TATA box binding factors (Wu et al., 1987; Wu and Berk, 1988; Simon et al., 1987; Williams et al., 1989), E2F (Thalmeier et al., 1989; Heibert et al., 1989), E4F1 and ATF (Lee and Green, 1987; Lee et al., 1987; Lin and Green, 1988; Pei and Berk, 1989) and CCAAT box binding factors (Williams et al., 1989).

Given these data, two basic mechanisms for E1A stimulation have been proposed. E1A could act directly by binding to a variety of transcription factors and deploy a potent activating region to stimulate transcription, a proposition supported by the domain exchange experiments of Lillie and Green, (1989). Alternatively, E1A could act to increase the quantities of active cellular transcription factors. Again evidence exists to support this proposition as increased levels of functional TFIIID (Hoeffler and Roeder, 1985; Yoshinaga et al., 1986), TFIID (Leong et al., 1988) and E2F (Raychaudhuri et al., 1987; Reidel et al., 1988; Babiss et al., 1989) have been detected following adenovirus infection. Of course, it is possible that E1A could act in either way on different promoters, though this proposition seems rather unlikely given that a 49 residue synthetic peptide corresponding to E1A domain 3 appears to be able to function autonomously to transactivate viral genes *in vitro* and *in vivo* (Pusztai et al., 1989). Thus the detailed mechanism of E1A action remains controversial.

1.5.3. DNA Binding Domains

The ability of proteins to bind DNA in a sequence

specific fashion is an essential pre-requisite for the regulation of transcription in both eukaryotic and prokaryotic systems (Maniatis et al., 1987). In principle, sequence information can be directly "read" from DNA by hydrogen bond formation in the major groove of B form DNA, without disruption of the double stranded structure (Seeman et al., 1976). Information can also be expressed, and presumably identifed, by means of structural inhomogeneities along the backbone of the DNA helix resulting from the specific sequence in question (Schleif, 1988).

Analysis of information derived from the published sequences of DNA binding proteins has allowed the identification of conserved structural motifs by which many proteins recognise DNA (Anderson et al., 1981; Fairall et al., 1986; Landschultz et al., 1988b; Johnson and McKnight, 1989).

1.5.3.1. Helix-Turn-Helix

The helix-turn-helix (HTH) structure was initially characterised through extensive analysis of the DNA binding domains of three prokaryotic regulatory proteins, the CRO and CI proteins of bacteriophage lambda and the catabolite activator protein (CAP) of E.coli. These proteins bind as dimers to sites which have dyad symmetry (Anderson et al., 1981; McKay and Steitz, 1981; Pabo and Lewis, 1982; Steitz et al., 1982). The essential structural components of their binding regions, which have been analysed in detail both by mutagenesis and crystallographic studies, are two short α-helices separated by a β-turn (Anderson et al., 1981; Jordan and Pabo, 1988; Otawinowski et al., 1988; Aggarwal et al., 1988). The carboxy-terminal helix, helix-3, interacts with the major groove, and is locked in place by hydrophobic interactions with helix-2 which is positioned over it relative to the DNA. Amino acid similarities between prokaryotic HTH proteins are less striking than their structural constancy (Pabo and Sauer, 1984), however, sufficient conserved residues are present to allow identification of new HTH motifs by sequence comparison. These have allowed the identification of HTH motifs in eukaryotic DNA binding proteins such as MATa1 and MATa2, the

products of the mating type locus in the yeast Saccharomyces cerevisiae (Scott and Weiner, 1984). In addition Langhorn and Scott, (1984) and Shephard et al., (1984) noted that the sequence similarities could be further extended to the products of Drosophila homeotic genes which were already known, through genetic analysis, to be intimately involved in the regulation of developmental processes (Wright et al., 1989).

The realisation that homeobox sequences share similarity with bacterial HTH proteins led to the prediction that these proteins would fulfill their regulatory function by forming sequence specific interactions with DNA. Although difficulties have attended attempts to link specific homeotic gene products to their appropriate sites on DNA, it is clear that homeobox-containing proteins localise in the nuclear compartment (DiNardo et al., 1985; Carrol and Scott, 1985), are capable of avid interaction with specific DNA sequences (Desplan et al., 1985; Beachy et al., 1988) and depend on the homeodomain for sequence-specific recognition of DNA (Desplan et al., 1988). That homeobox proteins can function as transactivators was recently confirmed by the identification of the POU proteins (Herr et al., 1988; Section 1.4.3.). Analysis of these proteins also revealed that the function of proteins that contain homeodomains is not confined to regulating developmental processes, as Oct-1 is found in almost every cell type (Fletcher et al., 1987), and no developmental role for it has yet been defined.

1.5.3.2. Zinc Finger Motifs

The zinc finger motif was originally proposed from studies on the RNA polymerase II transcription factor TFIIIA. Purification, cloning and sequencing of this factor, which binds to the internal control region of the 5S rRNA gene (Miller et al., 1985), revealed a sequential and ordered occurrence of cysteine (Cys) and histidine (His) residues ($Cys\text{-}N_2\text{-}Cys\text{-}N_{12}\text{-}His\text{-}N_3\text{-}His$) repeated nine times. These observations, coupled with earlier experiments that had shown that TFIIIA binds zinc (Hannas et al., 1983) prompted Fairall et al., (1986) to propose that each repeat

sequestered a single zinc ion via tetrahedral co-ordination with the spatially conserved Cys and His residues. The 12 amino acid loop between the invariant Cys and His pairs contains scattered basic and several conserved hydrophobic residues and is thought to be involved in specific interactions with DNA (Fairall et al., 1986). This type of zinc finger is also present in transcriptional regulatory proteins from yeast (Hartshorne et al., 1986; Stillman et al., 1988) and humans (Page et al., 1987; Kadonaga et al., 1987). These include the transcription factor Spl, which contains three zinc fingers that are known to be necessary and sufficient for DNA binding and require zinc to do so (Kadonaga et al., 1987).

A second type of zinc finger, related to, yet distinct from the Cys_2His_2 arrangement described above, has been observed in a wide range of DNA binding proteins (Weinberger et al., 1985; Miesfeld et al., 1986; Jeltsch et al., 1986; Green et al., 1986; Greene et al., 1986; Wray et al., 1987; Baum et al., 1987). This arrangement relies on clusters of 4 to 6 cysteine residues to coordinate the zinc ion. Like the Cys_2His_2 fingers these occur in 25 to 30 residue clusters that tend to be repeated. The best characterised members of this family of proteins are the mammalian hormone receptors. The rat glucocorticoid receptor requires zinc for DNA binding activity and substitution mutations that alter key cysteine residues result in loss of function (Freedman et al., 1988; Severne et al., 1988).

The mechanism by which zinc fingers establish sequence specific contacts with DNA has not yet been fully resolved. The model proposed by Fairall et al., (1986) suggested that the Cys and His residues form a stable scaffold through their interaction with zinc, allowing the residues between to loop out and form sequence specific contacts with the DNA. No evidence has come to light that contradicts this basic view though the small differences in DNA binding specificity between progesterone and oestrogen receptors have been shown to depend on differences in several non-conserved amino acids at the base of the finger regions (Mader et al., 1989).

1.5.3.3. The Leucine Zipper

A third conserved DNA binding motif has recently been identified and given the descriptive, if somewhat misleading, title "leucine zipper" (Landschultz et al., 1988b). In common with the zinc finger motif previously described, this DNA binding domain comprises two distinct parts, a structural region also involved in dimerisation, the zipper motif itself, and an adjacent DNA binding domain rich in basic amino acids. (Landschulz et al., 1988b). Both of these elements are required to allow specific protein-DNA interactions.

The leucine zipper was first proposed by McKnight's group (Landschulz et al., 1988b) as a result of studies on the protein C/EBP (section 1.5.2.3.). They noticed a heptad repeat of 4 leucine residues within a sequence that could form a stable α-helix with the leucine residues all present on one face. The next 30 residues contained a high proportion of basic amino acids. This whole structure was shown to lie within a fragment that contained the DNA binding activity of the entire protein (Landschulz et al., 1988a). A computer search revealed a similar leucine repeats in the amino acid sequence of the proto-oncogenes fos, jun and myc and the yeast regulatory protein GCN4, all of which were known to bind DNA as dimers (Hope and Struhl, 1987; Rauscher et al., 1988; Chiu et al., 1988; Sassone-Corsi et al., 1988; Lamph et al., 1988). Thus it was proposed (Landschulz et al., 1988b) that the leucine zippers form the interaction domain between monomers and the region containing the basic residues allows interaction of the dimer with DNA.

Details of the interaction between individual leucine zipper regions are still obscure, however they are known to be essential for the proteins' DNA binding activity (Landschulz et al., 1988a; Gentz et al., 1989; Turner and Tijan, 1989; Schuerman et al., 1989). The initial model for the interaction (Landschulz et al., 1988b) was that monomers bound in an antiparallel configuration with interdigitation of leucine residues on the helices stablising the interaction (thus the zipper metaphor). More recent studies have challenged this concept by demonstrating that the helices probably interact in a parallel configuration

(O'Shea et al., 1989; Landschulz et al., 1989; Gentz et al., 1989). These studies suggested that leucine zippers may adopt the coiled coil configuration found in fibrous proteins such as keratins, lamins, and myosin (Cohen and Parry, 1986), which classically depend for their structure, on the presence of a 4-3 repeat of hydrophobic amino acids. This arrangement is found in GCN4, jun and C/EBP but not in fos and myc, and a simple requirement for hydrophobic residues fails to explain the strict conservation of leucine residues.

The details of the interactions between leucine zipper motifs are essential to an understanding of the processes that control dimerisation, which in turn, is essential for an understanding of the DNA binding activities of this important family of regulatory proteins (see sections 1.5.2.1., 1.5.2.2. and 1.5.2.3.). GCN4 and C/EBP form only homodimers, while fos and jun can form both homodimers and heterodimers. Interestingly, the affinity with which the latter two proteins form dimers mirrors the affinity with which the completed dimer interacts with DNA. Thus fos/jun heterodimers form easily and bind DNA avidly, jun homodimers form less easily and bind DNA with less affinity, fos-fos homodimers are difficult to detect, and fos has low affinity for DNA (Nakabeppu et al., 1988; Turner and Tjian, 1989). These data suggested that the ability to dimerise and not the affinity of individual DNA binding domains regulates the affinity of protein-DNA interactions. This was confirmed by recent domain exchange experiments (Kouzarides and Ziff, 1989; Sellers and Struhl, 1989) which showed that linking the fos DNA binding region with the GCN4 zipper motif resulted in a dimer that bound the AP1 site with high affinity. Further corroboration of this model came from Neuberg et al., (1989), who exchanged the fos zipper motif with that of jun to create a protein which formed a homodimer and bound to the AP1 site with moderate affinity.

Potential leucine zipper motifs have been detected in many other proteins. These include transcriptional regulatory proteins such as Drosophila zeste protein and Oct-2 in which they are not required for binding to any known DNA recognition sequences (Johnson and McKnight, 1989) and proteins with functions unrelated to transcriptional

regulation such as voltage gated potassium channels (McCormack et al., 1989), glucose transporter proteins (White et al., 1989) and the F glycoproteins of paramyxoviruses (Buckland et al., 1989). In these examples the zipper motif is not flanked by a basic region. These data suggest that this motif has a much wider role in protein-protein interactions than the one involved in the transcriptional regulators thus far described.

The three categories of DNA binding motifs described above define three categories of eukaryotic DNA binding proteins which include approximately 80% of those proteins for which sequence data are available (Johnson and McKnight, 1989). Proteins that do not fall into these categories include heat shock transcription factor (Wiederrecht et al., 1988), CTF/NF1 (Santoro et al., 1988), HAP2 (Pinkham et al., 1987) and HAP3 (Hahn et al., 1988).

1.5.4. Activating Domains

Experiments in several systems have demons trated that at the simplest level, proteins or complexes that function as transcriptional activators have two functional surfaces. One of these is responsible for the appropriate positioning of the factor either through direct binding to DNA, or through protein-protein interactions. The other is required for the stimulation of transcription. This model was initially developed from work in prokaryotic systems in which mutants with activator proteins that bound DNA but failed to activate transcription were isolated (Guarente et al., 1982; Hochschild et al., 1983). The activating surfaces of these proteins were shown to consist of an amphipathic α-helix bearing solvent exposed, predominantly negatively charged, residues (Bushman and Ptashne, 1988). Further experiments showed that the organisation into independent DNA binding and transcriptional activation functions also held for eukaryotic activators. Brent and Ptashne, (1985) replaced the DNA binding domain of the yeast transcriptional activator GAL4 with that of the bacterial repressor Lex A, to produce a protein that, when expressed

in yeast, activated transcription of a gene bearing a Lex A operator upstream of its TATA box. These findings were confirmed using another yeast activator, GCN4 (Hope and Struhl, 1985, 1986)

Three experiments provided evidence that, like prokaryotic systems, one class of eukaryotic activators depended on the presence of local concentrations of acidic residues. Deletion analysis of GAL4 (Ma and Ptashne, 1987a) and GCN4 (Hope and Struhl, 1986) showed that the removal of acidic domains resulted in loss or reduction of transcriptional activity. Point mutations in GAL4 that increased transcriptional activity involved the incorporation of negatively charged residues (Gill and Ptashne, 1987). Finally, sequences that functioned as activating regions when attached to appropriate DNA binding domains were readily detected by random cloning of fragments of the E.coli genome (Ma and Ptashne, 1987b), and analysis of the active fragments showed that they often had the potential to form amphipathic α-helices (Ma and Ptashne, 1987b).

The implied requirement for secondary structure in activating regions was confirmed by the construction of two oligonucleotides, each encoding a 15 amino acid peptide which were attached to DNA encoding the binding domain of GAL4. One peptide was designed to form an α-helix with charged residues on one surface, in the other the same amino acids were arranged in scrambled order. Only the construct encoding the activating region in the form of the α-helix activated transcription in yeast (Hope et al., 1988).

The conservation of these acidic regions as activating domains was demonstrated in a series of experiments in which, given appropriate binding motifs, the well defined yeast transcriptional activator, GAL4, was shown to stimulate transcription in Drosophila larvae (Fischer et al., 1988), tobacco cells (Ma et al., 1988) and mammalian cells (Kakidani and Ptashne, 1988; Webster et al., 1988a)

A possible corollary to the basic premise that activating regions interact with other components of the transcriptional machinery to stimulate transcription is that in the absence of interactions with DNA, transcription factors would retain their capacity to interact with

components of the transcriptional machinery, sequestering them from transcriptional complexes and causing a general reduction in transcription. This model has proved accurate in both yeast and mammalian systems (Gill and Ptashne, 1988; Triezenberg et al., 1988; Sadowski et al., 1988). In addition, as noted previously, the HSV-1 protein Vmw65 contains an acidic, carboxy-terminal domain essential for its role as a transactivator (section 1.4.2.). This domain has been used in exchange experiments and shown to function as a potent transcriptional activating region (Sadowski et al., 1988).

While acidic activating regions were the first to be discovered and remain the best characterised, the sequences of several known transcriptional activators have been shown to lack regions containing significant net negative charge. Spl is a sequence specific transcriptional activator that binds, through three zinc finger motifs (see section 1.5.3.2.), to the motif GGGCGG found upstream of many eukaryotic genes. Deletion analysis of this factor has revealed four separate regions that contribute to transcriptional activation (Courey and Tjian, 1988). The two regions which are most potent as activators contain a high portion of glutamine residues (25% overall) they also have a low charge density. These regions appear to be functionally redundant, the presence of either conferring almost wild type activity on the protein (Courey and Tjian, 1988). The third activating region is only one tenth as active as the Gln rich regions, has a high charge density and a net charge of -6, but differs from other acidic activating regions in that increasing its net negative charge does not increase its ability to activate (Courey and Tjian, 1988). The fourth activating region is found at the C-terminus, it fails to function when present alone but appears to be necessary for full activity of the other regions (Courey and Tjian, 1988). Further evidence that glutamine rich sequences constitute bona fide transcriptional activating regions comes from experiments in which DNA encoding a 145 amino acid, glutamine rich region, from the Drosophila antennapedia protein was shown to substitute for the activation domains of Spl when linked to the Spl zinc fingers (Mitchell and Tjian, 1989). Inspection

of sequence data from other established or suspected transcription factors reveals glutamine rich regions in Drosophila ultrabithorax and zeste proteins (Mitchell and Tjian, 1989); in yeast HAP1, HAP2 and GAL4 (Pfeifer et al., 1989; Pinkham et al., 1987; Suzuki et al., 1988); and in mammalian factors Oct-1 (Sturm et al., 1988), Oct-2 (Scheidereit et al., 1988), jun (Bohmann et al., 1987), AP-2 (Imagawa et al., 1987) and serum response factor (SRF) (Norman et al., 1988), although the activation functions of these proteins have not yet been systematically defined.

A third type of activating region has recently been identified in CTF/NF1 (Mermod et al., 1989). Deletion analysis characterised it as a 100 residue region which contains 25% proline residues. It displays none of the features of the glutamine rich or acidic activating regions but will replace the activating regions of Sp1 when linked to the Sp1 DNA binding domain (Mermod et al., 1989). Again, proline rich regions have also been noted in other mammalian transcription factors (Imagawa et al., 1987; Struhl et al., 1988; Clerc et al., 1988; Norman et al., 1988), though analysis of their activity as functional activating regions is awaited.

1.5.5. Mechanism of Action of Transcriptional Activators

The endpoint of all processes mediated by transcriptional activators is the enhancement of RNA polymerase II mediated transcript initiation or elongation. Transcription is carried out by a multienzyme complex which, in addition to RNA polymerase II, contains a minimum of five basic factors; TFIIA, TFIIB, TFIID, TFIIE and TFIIF (Reinberg et al., 1987). These six factors are essential to form a pre-initiation complex that is resistant to the action of heparin, an inhibitor of transcriptional initiation (Reinberg et al., 1987). Kinetic studies have suggested the order in which factors associate with DNA to form the initiation complex (Samuels and Sharp, 1986). Initial binding requires factors TFIIA and TFIID which, along with RNA polymerase II, form a complex which is resistant to the presence of 0.02% sarkosyl, another

inhibitor of initiation (Davison et al., 1983). The situation regarding TFIIA is not as certain as for other factors since a requirement for this fraction has not been consistently demonstrated and there is significant disagreement relating to its molecular weight and properties (Reinberg et al., 1987; Samuels and Sharp, 1986; Egly et al., 1984). In contrast, TFIID has been well characterised as the TATA box binding factor. Its importance to transcription initiation has been demonstrated by its functional conservation between mammals (Sawadogo and Roeder, 1985) and yeast (Buratowski et al., 1988), though in terms of molecular weight the factors from these species vary between 120-140 kd in mammals (Samuels and Sharp, 1986) and 27 kd in yeast (Buratowski et al., 1988). Once TFIIA, TFIID and RNA pol II have bound DNA, rapid binding of the other factors required to form the heparin resistant complex occurs (Fire et al., 1984). This complex is competent for chain initiation and elongation (Bunick et al., 1982).

To function as transcriptional activators, proteins must act to enhance one or more of the steps described above. Evidence exists for at least two basic mechanisms by which this might be achieved. Activators could function indirectly, to alter the form of the DNA template and thus facilitate the formation of an initiation complex. The presence of CpG rich islands covering the regulatory regions of constitutively transcribed genes and the developement of nuclease hypersensitive sites around genes whose transcription has been induced (Gross and Garrard, 1988) is evidence for this mechanism, though questions of cause and effect in both situations have not been fully resolved.

A second mechanism by which transcriptional activators could function is by acting directly on the components of the initiation and elongation complexes. Evidence that this occurs is implicit in the multitude of demonstrations that transcriptional activators function in short term transfection assays and in cell free extracts. Insights into the mechanism by which transcriptional activators mediate their effects have begun to accumulate more recently with the availability of purified preparations and the sequences of the relevant factors.

TFIID has been identified as a target of several

transcriptional activators. The PRV IE protein appears to act by facilitating the interaction of TFIID with the promoter, a role that can be reproduced by pre-incubation of the DNA template with a purified preparation of the factor (Abmayer et al., 1988). ATF and GAL4 have also been shown to alter TFIID binding, Horikoshi et al., (1988a,b) showed that binding of either transactivator to sites upstream of the adenovirus E4 TATA box resulted in a qualitative alteration of the footprinting pattern of partially purified mammalian TFIID. These studies were complemented by functional analyses (Hai et al., 1988) which showed that transcription could be inhibited by an oligonucleotide containing the ATF binding motif added prior to TFIID, TFIIB and RNA polymerase II. Addition of the oligonucleotide after these factors left transcription unaffected, suggesting a transient requirement for the activator in the formation of a preinitiation complex.

Given the diversity of transcriptional activators thus far discovered, it would be surprising to find a single mechanism of action used by all. It is no surprise that other transactivators have been analysed and found not to act through TFIID. Earlier data from Roeder's group, who described the effect of ATF on TFIID using the adenovirus E4 TATA box, showed that no alteration in the TFIID footprint resulted from the binding of a gene specific transcription factor, USF, to the adenovirus major late promoter (Sawadogo and Roeder, 1985). Indeed, using this system, binding of TFIID alone protected a region from -40 to +35, the same nucleotides as were protected around the E4 TATA box, presumably by TFIID, in the presence of ATF (Horikoshi et al., 1988b). In a second model, using yeast TFIID (Schmit et al., 1989), the transcription factor Spl was shown not to affect the in vitro binding of this factor. This result is not directly comparable with those described above as while the yeast factor functionally replaces mammalian TFIID, the discrepancy in their molecular weights means that simple comparisons may not be directly informative.

Other data implicate RNA polymerase II as a target for activators. This enzyme comprises a large multiprotein complex consisting of 8-10 subunits ranging in molecular weight from 240,000 to 10,000 kd. The largest subunit is

highly conserved from yeast to man and has a carboxy-terminal domain consisting of the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser repeated 52 times in mice and 26 times in yeast. This carboxy-terminal domain has been proposed as a site for the action of transcriptional activators as its removal eliminates transcription and partial deletions cause graded reductions in activity (Allison et al., 1988, Bartolmei et al., 1988).

An elegant study by Allison and Ingles, (1989), provided insights into the role of the carboxy-terminal repeats of RNA polymerase II and their functional relationship with acidic activating regions. These workers made mutant yeast strains containing alterations in two proteins. Deletions or extensions were made to the RNA polymerase II carboxy-terminal repeats and deletions were made to the acidic regions encoded by the transcriptional activator GAL4. Analysis of yeast strains combining mutations in both proteins revealed that wild type GAL4 could complement partial deletions of repeats from RNA polymerase II without loss of function. Mutants with deletions of acidic residues and the RNA polymerase II carboxy-terminal repeats had considerably reduced activity. Conversely, activity in mutants with large deletions in the activating region of GAL4 such that its stimulating activity on wild type RNA polymerase II was reduced, could be rescued by mutations which resulted in an increase in the number of repeats at the C-terminus of RNA polymerase II. Thus a functional connection can be drawn between the acidic regions of activators and the carboxy-terminal repeats of RNA polymerase II, though no information as to the mechanism by which they are connected can be implied.

Even from the evidence available to date, it is clear that the regulation of eukaryotic gene expression is a complex and flexible system. Current knowledge provides a reasonable picture of the first level of complexity in gene regulation, that of protein-DNA interactions. In a few systems this rather narrow focus has been expanded to show the importance of protein-protein interactions which will provide a second level of complexity. These interactions can be either direct, with components of the transcriptional

machinery as apparently occurs in the case of ATF and TFIID (Horikoshi et al., 1988a), or indirect, with the formation of multi-protein complexes as in the case of the Vmw65-Oct-1 interaction (Preston et al., 1988; Gerster and Roeder, 1988) and the HAP2/3/4 interaction in yeast (Forsburg and Guarente, 1989).

Surprising complexity exists even at the level of protein-DNA interactions with multiple proteins interacting with the same DNA motif and multiple DNA motifs interacting with the same protein. Both of these phenomena can be viewed as increasing the flexibility of the transcriptional regulatory machinery. Further advances in the understanding of gene expression will no doubt produce more surprises, but hopefully also a greater understanding of the mechanisms that control this vital process.

1.6. Aims of the Project.

The initial aim of this project was the analysis of VZV orfs by expression in heterologous systems. The first orf chosen for study was number 66 which encodes a putative protein kinase. The system chosen for its expression was the HSV-1 mutant tsK under the control of HSV-1 IE sequences (see Appendix 2). This course of study proved fruitless in that no product of orf66 could be detected on SDS-PAGE. After the first year, preliminary analysis was undertaken on two other VZV orfs. One was the VZV dPyK gene which was expressed using the plasmid pKK233-2 (see Appendix 1), the other involved investigation of the regulation of VZV IE gene expression with a view to analysing the function of orf 10 the proposed homologue of the HSV protein Vmw65. For reasons that are now apparent (Appendix 1) successful expression of the VZV dPyK gene did not result in detectable TK activity however investigation of the regulatory sequences of the VZV major IE gene yielded interesting data. First the site of mRNA initiation was mapped. Second, functional analyses were performed to determine the sequences that are important in the stimulation of transcription. Third, protein binding assays were undertaken in an attempt to identify the protein species responsible for transcriptional stimulation. These data

revealed that, like HSV-1, HSV-2 and PRV, the expression of the major IE gene of VZV is responsive to stimulation by the HSV-1 protein Vmw65. Analysis of VZV orf10, the homologue of Vmw65 was then undertaken to determine whether it possessed transcriptional stimulating activity. The results of these analyses are presented.

2. MATERIALS

2.1. Viruses

HSV-1 viruses used in this study were all derived from strain 17(syn$^+$) (Brown et al., 1973) including the mutant tsK (Marsden et al., 1976).

The VZV strain used was that described by Dumas et al., (1981) which was propagated from stocks held by Dr. D. Dargan.

2.2. Tissue Culture Media

ETC_{10} - Glasgow Modified Eagles Medium (GMEM) (Busby et al., 1964, supplied by Gibco) supplemented with 100 units/ml penicillin, 100ug/ml streptomycin, 10% tryptose phosphate and 10% calf serum.

DC_5 - Dulbecco's Modified Eagles Medium (DMEM) (supplied by Gibco) supplemented with 100 units/ml penicillin, 100ug/ml streptomycin, 2.5% foetal calf serum (FCS) and 2.5% calf serum.

EF_{10} - GMEM supplemented with 100 units/ml penicillin, 100ug/ml streptomycin, and 10% FCS.

For viral titrations infected cells were overlaid with GMEM supplemented as above and with 0.6% Noble Agar.

2.3. Tissue Culture Cells

BHK-21 clone 13 cells (Macpherson and Stoker, 1962) were used for growth and titration of HSV-1 and in transient transfection assays.

WS HeLa cells (originally obtained from Dr. W. Schaffner, Zurich) were used for transient transfection assays.

Flow HeLa Cells (Flow Laboratories) were used in the production of nuclear extracts.

Vero Cells (Flow Laboratories) were used for transient transfection assays.

Human Foetal Lung (HFL) cells were originally supplied by Dr. B. Carritt and used for the propagation of VZV.

2.4. Bacterial Strains

Escherichia coli K12 DH1 (*recA1*, *nalA*, r_R^-, m_R^-, *endoI*$^-$, B$^-$, *relA1*) (Hanahan, 1983) was used for routine plasmid construction and amplification.

Escherichia coli W3110 *LacI*^{q}, L_8 (Brent and Ptashne, 1981) was used for the induced expression of VZV orf36.

2.5. Bacterial Culture Media

Bacteria were routinely grown in L. Broth [1% (w/v) NaCl, 1% (w/v) bactopeptone, 0.5% (w/v) yeast extract] and L. Broth agar [L. Broth plus 1.5% (w/v) agar].

2.6. Plasmids.

pVZVSstf Terminal SstI fragment of the short repeat of VZV in the vector pAT153 (Davison and Scott, 1983).

pVZVKpnj KpnI fragment of the short unique region of VZV containing the putative protein kinase gene. (Davison and Scott, 1983)

pFJ3 pCH110 (BIOLABS) with polylinker of pUC18 added at residue 4080 (Dr. F. Rixon, pers. comm.)

pBLW2 CAT gene with HSV-2 IE 5 terminator sequences in pUC8 (Gaffney *et al.*, 1985).

p140cat VZV ORF62 upstream region from -1146 to +55 inserted into pCAT (McKee *et al.*, in press)

pFS8 HSV-1 TK gene under the control of SV40 E gene regulatory sequences (Campbell *et al.*,

1984)

pIE3CAT HSV-1 IE gene 3 regulatory sequences (-331 to +26) fused to the CAT gene in a vector derived from pBLW2 (Stow et al., 1986).

pRR55 IE promoter and enhancer sequences of HCMV strain AD169 (Fickenscher et al., 1989). Kindly provided by Dr. R. Ruger

pl2S Adenovirus 5 E1A gene and control sequences with the 12S cDNA replacing coding sequences. Kindly provided by Dr. N. Jones.

pl3S Adenovirus 5 E1A gene and control sequences with the 13S cDNA replacing coding sequences. Kindly provided by Dr. N. Jones.

pMC1 Coding and regulatory sequences of Vmw65 inserted as a 2.7kb fragment into pUC9. (Campbell et al., 1984).

pTZORF10 1290bp fragment containing VZV orf10 cloned into pTZ18 (Pharmacia). (McKee et al., in press).

pMCORF10 Coding region of VZV orf10 cloned into pMC1.in17 (Ace et al., 1988; McKee et al., in press).

pGEMTIF HSV-1 gene encoding Vmw65 cloned under the control of the T7 promoter in pGEM2.

A494*23 A 360bp fragment containing the HSV-1 IE-3 regulatory sequences inserted into the HSV-1 BamHI p fragment within the coding sequences of the TK gene. Provided by Dr. C.M. Preston.

2.7. Oligonucleotides

Oligonucleotides were synthesised using a Biosearch 8600 DNA synthesiser by Dr. J. McLaughlin or provided by Dr. N. Jones. Sequences of the oligonucleotides used are given in Appendix 3.

2.8. Chemicals

Chemicals used were of the highest purity available and were obtained from Sigma Chemical Co., BDH Chemicals or an alternative supplier. Other suppliers included Kochlight Ltd. (boric acid, acrylamide, caesium chloride, sodium hydroxide, trichloracetic acid [TCA]) and Pharmacia Fine Chemicals (Sephadex G50).

2.9. Radiochemicals

Amersham and NEN Dupont supplied all radiochemicals used.

2.10 Commonly Used Buffers

EEB (electroelution buffer)
40mM Tris, 5mM sodium acetate, 1mM EDTA.

TBE 90mM Tris, 90mM Boric acid, 1mM EDTA, pH8.3

TBELB TBE loading buffer
50% (v/v) 1xTBE, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue.

SSC 150mM NaCl, 15mM trisodium citrate.

2.11 Enzymes

DNAase, RNAase and Lysozyme were supplied by Sigma; Proteinase K, T4 polynucleotide kinase and T4 ligase were from Boeringer Mannheim, most restriction endonucleases were from Bethesda Research Ltd. or New England Biolabs though AflIII came from Northumberland Biologicals. Reverse Transcriptase, isolated from avian myloblastosis virus came from Life Sciences Inc.

3. METHODS

3.1. Tissue Culture

Baby hamster kidney BHK-21 (C13) cell monolayers were grown in ETC_{10} medium at 37°C, on 175cm^2 (Nunclon) flasks, in an atmosphere of 5% CO_2, 95% air. Cultures were passaged every 2 to 3 days. This was achieved by removal of growth medium, addition of 10 ml of trypsin/versene (1:1 v/v) and incubation until cells detached from the flask. ETC_{10} (10ml) was added and the cells resuspended by pipetting. This suspension was used to seed further monolayers.

Monolayer cultures of human foetal lung (HFL) cells were grown in EF_{10} medium at 37°C on 175cm^2 (Nunclon) flasks in an atmosphere of 5% CO_2, 95% air. The cultures were passaged every 3 to 4 days as described above.

HeLa cells (obtained from Dr. R. D. Everett) were grown in Dulbecco's Modified Eagles Medium (DMEM) (supplied by Gibco) supplemented with 100 units/ml penicillin, 100ug/ml streptomycin, 2.5% foetal calf serum (FCS) and 2.5% newborn calf serum. They were routinely passaged in 175cm^2 (Nunclon) flasks and harvested as described above.

Vero cells (obtained from Dr. R. D. Everett) were grown in EF10 medium at 37°C on 175cm^2 flasks in an atmosphere of 5% CO_2, 95% air. The cultures were passaged every 2 to 3 days as described above.

3.2. VZV Stocks and Propagation

The VZV strain used for these studies was that described by Dumas *et al.* (1981), which was kindly provided by Dr. D. Dargan. Stocks were produced by infecting subconfluent HFL monolayers with VZV-infected HFL cells. These were incubated at 37°C until a cytopathic effect (cpe) of 60%-80% had developed. This normally occured after 4-5 days but infected cultures could be subcultured as described in section 3.1. and the infection allowed to continue until an adequate level of cpe developed. Cells were harvested using trypsin and versene as described above, (section 3.1.) and $^1/_6$ of the product of one 750 ml flask used to continue the infection in another flask of subconfluent cells. The

rest of the cells were pelleted (1500 rpm for 2min), resuspended in EF_{10}, with 10% dimethylsulphoxide (DMSO) and a further 10% FCS added. Cells were then stored at -140°C.

3.3. Mapping of mRNA 5'terminus

3.3.1. Extraction of Cytoplasmic RNA

For RNA preparation cells were grown on 90mm plates until 80% confluent. HFL cells, infected with VZV, were harvested as described (section 3.2.) and approximately $5x10^5$ cells were added to each plate. The plates were incubated at 37°C for 24 hr. Medium was then decanted, the cells washed in ice cold PBS and monolayers scraped into ice cold PBS. This suspension was centrifuged at 1000g for 5 min at 4°C. Cell pellets were resuspended in 5ml of lysis buffer (0.2M Tris HCl pH 8.5, 0.14M NaCl, 2mM $MgCl_2$, 10ug/ml cycloheximide, 0.5% NP40), pipetted 4 times through a 10ml pipette and centrifuged at 1000g for 3 min at 4°C. The supernatant was transferred to a flask, at room temperature, containing 15ml 3mM Tris HCl (pH 8.5), 2mM EDTA, 0.2% SDS and 15ml phenol/chloroform. The contents were mixed by swirling then left for 10 min before centrifugation at 1000g for 10 min at 20°C. This procedure was repeated two further times after which the supernatant was added to 15ml of chloroform mixed and centrifuged for 5 min at 1000g. NaCl (0.1M) was added to the supernatant from this final extraction and RNA precipitated overnight at -20°C after addition of 2 volumes of ethanol. The RNA was then pelleted by centrifugation at 1000g for 10min at 4°C, washed in ethanol, dried and dissolved in 150ul of distilled water.

3.3.2. Oligonucleotide Purification

Oligonucleotides were produced by Dr. J. McLaughlan using a Biosearch 8600 DNA Synthesiser. On receipt they could be stored at -20°C. Before purification protecting groups were removed by incubating the sample at 55°C for 5 hr. The samples were then frozen, dried under vacuum and redissolved in sample loading buffer (90% v/v deionized

formamide and 1xTBE). Oligonucleotides were then loaded onto a denaturing acrylamide gel (15% with 4% crosslinker) (section 3.2.7.) and run at 35 W. Separate dye tracks were employed to monitor progress and the run stopped after the xylene cyanol band had progressed past 7cm. The oligonucleotide bands were visualised by the shadow casting technique, in which the gel was removed from the plates and placed on a fluorescent thin layer chromatography plate. An ultraviolet lamp was used to observe fluorescent quenching and the desired oligonucleotide, always the slowest moving dark band, was excised. The gel fragment was then diced and incubated overnight at 45°C in gel elution buffer (GEB) (0.5M sodium acetate, 0.1% w/v SDS, 2mM EDTA, 20mM Tris HCl (pH7.5)) with. Samples were then filtered through a Whatman GF/C filter, precipitated in 2.5 volumes of ethanol at -20°C for 2 hrs, centrifuged at 36,900g for 20 min, washed in ethanol dried and resuspended in 50ul of water.

3.3.3. Generation of Double-Stranded Oligonucleotides.

If double stranded oligonucleotides were required they were annealed by mixing 25ul of each preparation in a solution containing 0.3M sodium acetate, 2mM EDTA and 40mM Tris HCl pH7.5. These were incubated at 50°C for 1 hr, 37°C for 1hr and at room temperature for 1 hour. After this the annealed oligonucleotides were precipitated in 2.5 volumes of ethanol for 2hr at -20°C, centrifuged at 36900g for 20 min, washed in ethanol and dried.

The concentration of oligonucleotides was established by comparison of samples with previously quantified oligonucleotides. Double stranded and single stranded oligonucleotides were separated on a 14% non-denaturing polyacrylamaide gel (section 5. 3. 2.) and samples were compared by fluorescent quenching.

3.3.4. 5' [^{32}P] Labelling of Probes

Oligonucleotide probe (50ng) was dissolved in kinase buffer (70mM Tris HCl pH7.6, 10mM $MgCl_2$, 5mM DTT), 50uCi of [^{32}P]- ATP plus 2 units of T4 polynucleotide kinase were added and the mixture incubated at 37°C for 1hr. The

radiolabelled sample was loaded onto a 5% non-denaturing acrylamide gel and run at 250V for 2hr as described in section 3.3.2. The appropriate band was cut out, chopped into small pieces and incubated overnight in 600ul of GEB at 45°C. After this incubation the supernatant was decanted and purified by passage through a Sephadex G50 column and collected in 250ul samples. The five samples showing the highest levels of radioactivity were selected, to these was added lug of poly(dI).poly(dC) prior to precipitation in ethanol for 30min at -70°C. Following centrifugation at 11,600g for 10 min, washing in ethanol and drying, the pellets were dissolved in 10ul to 100ul of water and subjected to Cerenkov counting.

3.3.5. Primer Extension Analysis.

Assays were carried out as described by Inoue and Cech (1986). 10ug of RNA was annealed to a 5' end labelled oligonucleotide primer. The primer, a 29-mer, (5'-GGGGTGTAGAGCGCTGCATCGGCGGCGAT '3) was designed to be complementary to the N-terminus of the VZV orf62. Hybridisation was initiated at 90°C in hybridisation buffer containing 50 mM Tris pH 8.3 and 40 mM KCl. Thereafter the temperature was allowed to decrease to 45°C over one hour. $MgCl_2$ was added to 5 mM and each deoxynucleoside triphosphate to 500 uM. This mixture was incubated with 10 units of reverse transcriptase for 30min and compared with size markers using denaturing polyacrylamide gel electrophoresis.

3.3.6. Nuclease S1 Analysis.

Approximately 5ng of oligonucleotide 5' end labelled with [^{32}P]- ATP was precipitated with 10ug of cytoplasmic RNA. The resulting pellet was resuspended in 20ul of 90% v/v deionised formamide, 0.4M NaCl, 40mM PIPES (pH6.8), 1mM EDTA and heated at 90°C for 5min. Samples were transferred rapidly to a water bath at 58°C and incubated for 16hr. They were then placed on ice. 4000 units of S1 nuclease in the appropriate buffer were added to each sample to give final concentrations 25mM NaCl, 30mM sodium acetate (pH4.5),

0.1mM $ZnSO_4$ in a volume of 200ul.

The samples were incubated at 37°C for 90min. EDTA (pH 7.5) and sodium acetate (pH 7.0) were then added to final concentrations of 20mM and 0.3M respectively. The samples were phenol/chloroform extracted, ethanol precipitated and electrophoresed on a denaturing polyacrylamide gel alongside G and G + A sequence tracks.

3.3.7. G and G + A Sequencing

G and G + A sequences were derived to provide marker tracks for both nuclease S1 analyses and DNAase I footprinting assays. The method followed was that of Maxam and Gilbert (1977). DNA analysed was uniquely end labelled using either T4 kinase or T4 polymerase, sections 3.2.3. and 3.6.4. respectively.

Radiolabelled DNA was resuspended in 0.1 mM NH_4OH, 3ug of sonicated calf thymus DNA was added and the total volume adjusted to 10ul with deionized water. Samples were then heated to 90°C for 1 min and chilled on ice.

To cleave at G residues 200ul of G buffer (50mM sodium cacodylate pH8.0 and 1mM EDTA) and 1ul of DMS were added and the mixture incubated at 20°C for 10 min. The reaction was stopped by the addition of G stop (1.5M ammonium acetate pH7.0, 1M 2-mercaptoethanol, 40ul carrier DNA). DNA was precipitated at -70°C in ethanol and pelleted by centrifugation for 10 min at 11,600g. The supernatant was then decanted and 250ul of 2M ammonium acetate/0.1M EDTA and 750ul ethanol added. It was precipitated at -70°C and centrifuged as above. The resulting precipitate was washed with ethanol and dried.

Cleavage at G + A residues was achieved by adding 10ul of water and 2ul of PIP-Formate pH4 (9.5 ml H_2O, 0.45ml 88% formic acid, 15ul piperidine) and incubating at 37°C for 25min. The reaction was then frozen, lyophylised, dissolved in 20ul of water and again frozen and lyophylised.

Ammonium hydroxide (5M) was then added to both samples, they were lyophylised, and 100ul of 1M piperidine added prior to incubation at 90°C for 30min. The samples were transferred to 1.5 ml reaction tubes containing 100ul of 2M ammonium acetate/0.1mM EDTA, ethanol precipitated, washed

and dried. Samples could be stored in this condition ready for use.

3.3.8. Denaturing Polyacrylamide Gels.

A 50ml volume of acrylamide solution was prepared, containing 8% or 10% acrylamide (diluted from a stock of 29% acrylamide, 1% N,N'methylenebisacrylamide, 7M urea), 21ml of 10M urea, 0.55xTBE, 300ul of 10% ammonium persulphate (APS) and 50ul TEMED. This solution was poured into a mould (230mm/450mm/0.35mm). After polymerisation the gel was pre-run at 40W for 1hr prior to loading. The samples were dissolved in 80% deionized formamide, 0.55xTBE, 1mM EDTA, 0.1% w/v xylene cyanol and 0.1% bromophenol blue, and denatured by heating to 100^{o}C for 3min before loading. Electrophoresis was performed in 0.55xTBE at 40W for approximately 2hr.

3.4. Construction, Preparation and Characterisation of Plasmid DNAs.

3.4.1. Restriction Enzyme Digests.

DNA was digested in a final volume of between 10ul and 50ul in the reaction conditions specified by the manufacturer of the enzyme used. The number of units of enzyme added was dependent on the activity of the enzyme and the amount of DNA present. Reaction mixtures were generally incubated at 37^{o}C for 3hr.

Partial cleavage to produce linear molecules was achieved by incubating 2ug of DNA with 2 units of enzyme in the presence of 10ug/ml to 500ug/ml ethidium bromide to identify conditions that produced a maximum of singly cut molecules.

3.4.2. Separation of Fragments by Non-denaturing Gel Electrophoresis.

Agarose gels: 200ml horizontal slab gels (260mm/160mmx7.5/mm) containing 0.5 to 1.5% (w/v) agarose were electrophoresed in 0.5xTBE plus 0.5ug/ml ethidium

bromide, for approximately 16hr at 50V. Samples were loaded in 10% glycerol, 0.5xTBE and 20ug/ml bromophenol blue.

: 50ml (100mm/70mm/7mm) gels were also used. These gels were electrophoresed in TBE at 40V for 1hr. Samples were loaded as for the horizontal slab gels and bands visualised by the addition of lug/ml ethidium bromide and subsequent UV transillumination.

Polyacrylamide gels: Vertical non-denaturing polyacrylamide gels were used for resolving DNA fragments smaller than 0.5kbp and analysis of DNA-protein interactions in gel retardation assays. A solution of 50ml containing 5 to 10% acrylamide (diluted from a stock of 29% acrylamide and 1% methylenebisacrylamide) and 0.5xTBE was prepared and 0.7ml of 10% APS and 50ul TEMED added immediately prior to pouring into a prepared gel sandwich (260mm/160mm/1mm). Acrylamide was allowed to polymerise and then samples were applied in 5% glycerol, 0.5xTBE and 0.1% bromophenol blue. Electrophoresis was carried out in 0.5xTBE at 160-250V for 2-4hr. Bands were visualised either by soaking the gel in lug/ml ethidium bromide prior to UV illumination, or when DNA bands were radiolabelled, by autoradiography of the wet or dried gel.

3.4.3. Purification of Fragments from Gels.

Agarose gels: a slice of agarose containing the required DNA fragment was removed from the gel. The DNA was isolated from the agarose by electroelution in 1xEEB at 20mA/sample for 1-2hr using an apparatus designed for this purpose. The DNA was removed in 200ul EEB and purified by phenol/chloroform extraction and precipitation with an equal volume of isopropanol at room temperature in the presence of 5ug of carrier, E.coli ribosomal RNA (rRNA). Carrier was not added if the DNA was for use in phosphatase or kinase reactions.

Polyacrylamide gels: gel slices containing DNA fragments were cut into small pieces and incubated overnight in 600ul of GEB at 45°C. The sample was then centrifuged for 2min at 11,600g and the supernatant retained. The acrylamide pellet was washed with a further 400ul of GEB and

centrifuged as before. The supernatants were pooled and filtered through Whatman GF/C paper in a 2ml syringe. The DNA was then precipitated at -20°C for 1-3 hr by the addition of 2 volumes of ethanol.

3.4.4. Ligation of DNA Fragments into Plasmid Vectors.

Ligations were performed using 50ng of linearised vector, 200-500ng of purified fragment and 1 unit of T4 ligase in 20mM Tris HCl (pH 7.6), 10mM $MgCl_2$, 10mM DTT and 0.6mM ATP in a 4ul reaction at 15°C for 16hr.

If the cleaved ends of the vector DNA were compatible with each other, reannealing of the linearised vector was prevented by pretreatment with calf intestinal phosphatase either in 50mM Tris HCl (pH 8.5) or in the same conditions as the restriction enzyme digest. Purification of fragments was either by gel electrophoresis (section 3.4.3.), or phenol/chloroforn extraction and precipitation.

Staggered cut termini of DNA fragments were converted to blunt ends by treatment with T4 DNA polymerase. Reactions contained 0.5-1.0ug DNA, 33mM Tris HCl (pH 7.8), 66mM potassium acetate, 10mM magnesium acetate, 100ug/ml BSA, 200uM dCTP, dGTP, dTTP and dATP plus 4 units of T4 DNA polymerase, and were incubated for 1hr at 37°C.

Phosphorylated restriction enzyme site linkers were inserted into vectors by the same procedure as DNA fragments, each ligation using 0.15ug of linker.

3.4.5. Transformation of E.coli.

10ml of L.Broth was inoculated with 10ul of a glycerol stock of DH-1 cells and incubated at 37°C overnight. 1ml of this culture was added to 90ml of L.Broth and the mixture shaken at 37°C until the OD_{600} was 0.2 (normally for 2.5 hours). After cooling on ice for 10min the culture was centrifuged at 1000g for 10min at 4°C. Bacterial pellets were resuspended in a total of 50ml of ice cold 100mM $CaCl_2$ and incubated on ice for 1hr. This mixture was centrifuged as before and E.coli resuspended in 1ml of ice cold 100mM $CaCl_2$. 2ul of ligation mix was added to 100ul of $CaCl_2$ shocked cells and incubated on ice for 1hr. The mixture was heated at 42°C for 1 min and added to 2ml of L.Broth. These

cultures were agitated at 37°C for 90 min and 150ul samples were spread on L.Broth agar plates containing the appropriate antibiotic. Plates were incubated at 37°C overnight.

3.4.6. Analysis of Transformed E.coli Colonies.

Colonies were picked from agar plates into 2ml of L.Broth plus antibiotic and shaken at 37°C for 7hr. Chloramphenicol was added to a final concentration of 25ug/ml and incubation continued overnight at 37°C. The following day the E.coli cells were pelleted at 2,900g for 5min and resuspended in 100ul of STET (8% w/v sucrose, 5% v/v Triton X100, 50mM EDTA, 50mM Tris HCl pH 8.0) containing 1mg/ml lysozyme. Following incubation at 100°C for 1min the mixture was centrifuged at 11,600g for 10min. The supernatant was precipitated by addition of 100ul of isopropanol at -20°C for 1 hr. Following centrifugation at 11,600g for 10min the pellets were dried and resuspended in 33ul of water.

These DNA samples were generally assayed for large insertions or deletions by screening for increased or decreased size compared to vector DNA. Further analysis was carried out by restriction digestion of samples.

3.4.7. Large Scale Plasmid DNA Preparations.

Two methods were used for the large scale preparation of plasmid DNA. For routine preparations the "hard lysis" procedure, an adaptation of the method of Guerry et al. (1973) was used, however, if the plasmid was required for short term transfection assays in HeLa cells further purification by CsCl banding was required and the "maxi-boiling" technique of Holmes and Quigly (1981) was used.

Hard Lysis: A single colony or 10ul of glycerol stock of bacteria containing the appropriate plasmid was added to 10ml of L.Broth plus antibiotic and incubated at 37°C overnight. From this stock 0.5 ml was taken and used to inoculate 350ml of L.Broth containing antibiotic and the

culture was shaken at 37°C for 8hr. Chloramphenicol was then added to a final concentration of 25ug/ml and incubation continued overnight. The culture was then centrifuged at 4,000g for 10min. After decanting the supernatant, the pellet was suspended in 5ml of 25% w/v sucrose, 50mM Tris HCl (pH 8.0), and 2.5ml of lysozyme, freshly prepared at 10mg/ml, was added. The samples were mixed and left on ice for 30 min. 2ml of 250mM EDTA (pH 7.5) was added and, after a further 5min on ice, 1.5 ml of 5M NaCl and 1.5 ml of 20% w/v SDS were mixed with the extract. After incubation on ice for a further 2hr, the preparations were centrifuged at 36,900g for 1hr at 4°C. The supernatant was then subjected to two extractions with phenol/chloroform, one with chloroform alone and precipitated with two volumes of ethanol at -20°C. The precipitate was pelleted by centrifugation at 1500g for 15min and dissolved in 10ml of 20mM Tris HCl, pH 7.5, 100mM NaCl, 1mM EDTA plus 10ug/ml RNAase. After incubation at 37°C for 4hr, approximately 2mg of proteinase K was added and samples maintained at 37°C overnight. The proteinase K was removed by two phenol/chloroform extractions plus one chloroform extraction. Sodium acetate was added to a final concentration of 0.3M and DNA was precipitated by addition of 0.5 volumes of isopropanol for 2hr at room temperature. After centrifugation at 1500g for 15 min pellets were washed with ethanol, dried and dissolved in 500ul of water.

"Maxi-Boiling" Technique. Bacterial cultures were prepared as above. Cells were pelleted and resuspended in 17ml of STET per 350ml culture. 2ml of 10mg/ml lysozyme in STET was added for 1min incubation prior to boiling the suspension for 45sec. The lysate was cleared by centrifugation at 36,900g for 40min at 4°C and DNA precipitated from the supernatant by addition of 0.9 volumes of isopropanol. After pelleting by centrifugation at 1500g for 10min at room temperature the drained pellets were resuspended in TE (10mM Tris HCl pH 7.5, 1mM EDTA pH 7.5), CsCl was added to give a final density of 1.6g/ml and ethidium bromide added to a final concentration of 0.5mg/ml. The solution was incubated on ice for 15min and cleared by centrifugation at 1,500g for 10min at 4°C. The DNA was

banded by centrifugation at 128,500g for 16hr at 15°C in a TV865 rotor. DNA was visualized by daylight or longwave UV illumination and the lower, supercoiled, band recovered with a large bore needle and a syringe, after removal of the upper linearised, chromosomal DNA band. The DNA was then extracted twice with butan-1-ol (TE saturated), dialyzed against TE at room temperature for 3hr, then treated with RNAase and proteinase K, purified and dissolved as described above.

3.4.8. Estimation of DNA Concentration.

A series of dilutions of plasmid DNA, linearised with an appropriate restriction enzyme was prepared. The samples were electrophoresed beside DNA of known concentration and photographed on polaroid 655 film under UV illumination. Concentration of DNA was estimated from densitometric traces on a negative film.

3.5. Functional Assays of Plasmid Constructs

3.5.1. Transfection of Plasmid DNA into BHK Cells.

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

Mixtures were prepared containing plasmid DNAs to a total of 9ug, and deionized water added to a total volume of 173ul, 200ul of 2xHeBS (260mM NaCl, 9.8mM KCl, 1.6mM Na_2HPO_4, 11mM D-glucose, 42mM HEPES, pH7.05) was then added and the solution vortexed. Finally 27ul of 2M $CaCl_2$ was added and samples again mixed by vortexing. The tubes were allowed to stand at RT for 10min while the medium was removed from 80% confluent cell monolayers grown in 50mm diameter petri dishes. The precipitates were added dropwise to the cell monolayers and incubated at 37°C for 45min with intermittent shaking.

5ml of ETC_{10} medium was added to each monolayer and the incubation continued for a further 3-4hr at 37°C. The medium was then removed from the cells and 25% v/v DMSO in 1x HeBS was applied at room temperature for 4 min (Stow and

Wilkie, 1976). The DMSO was then removed and cells were washed twice with ETC_{10}, a further 5ml of ETC_{10} was added and monolayers were incubated at 37°C for 16hr.

3.5.2. Transfection of Plasmid DNA into HeLa cells.

Freshly split cells were seeded at 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). Transfections used 12ug of pl40CAT or its derivatives, 2ug of pFJ3 and 4ug of pMCl; pUCl8 was used to equalise amounts of DNA within experiments. Deionised water was added to the plasmids to a final volume of 140ul, 160ul of HBS (280mM NaCl, 50mM Hepes, 1.5mM Na_2HPO_4 pH7.12) and 20ul of 2M $CaCl_2$ were then added, each step being followed by brief vortexing of the samples. After standing for 30 min the precipitate was added dropwise to the cell monolayers which were then incubated at 38.5°C. The cells were washed after 18hr, fresh medium added and, after a further 24hr at 38.5°C, extracts were prepared.

3.5.3. Preparation of Cell Extracts.

Medium was removed and monolayers were washed with ice cold PBS. 2ml of ice cold TEN (100mM NaCl, 50mM Tris HCl (pH 7.5), 10mM EDTA) was then added, the monolayers were scraped off the plates and transferred to 15ml Falcon tubes. The cells were centrifuged at 1500g for 1 min, resuspended in 75ul 0.2M Tris HCl (pH 7.5), lysed by sonication, transferred to 1.5ml vials and centrifuged at 11,600g for 2 min to remove debris. The cell extract supernatants were stored at -70°C.

3.5.4. Chloramphenicol Acetyl-transferase Assays of Transfected Cells.

CAT assays were performed using the method of Gorman (1982). Assay mixtures containing cell extract (section 3.5.3.), adjusted on the basis of beta-galactosidase

activity and diluted to 25ul with 0.2M Tris HCl (pH7.5), 1ul 50mM acetyl co-enzyme A, 14ul water and 0.5ul [^{14}C]-chloramphenicol (45uCi/mmol), were incubated at 37^{o}C for 1hr. The reaction was stopped by extracting the chloramphenicol and its acetylated products in 200ul of ethyl acetate. The ethyl acetate was removed under vacuum, and the pellets dissolved in 20ul of ethyl acetate before spotting onto thin layer chromatography plates. After running in 95% v/v chloroform/methanol, plates were air dried and autoradiographed. The amount of radioactivity in the chloroamphenicol and its 3-monoacetylated product was determined by scintillation counting, enabling the percentage conversion of substrate to product to be calculated. The amount of radioactivity as 1-acetylated chloramphenicol was not significant.

3.5.5. Preparation of Cell Extract for TK Assay

Medium was removed from cell monolayers which were then washed with ice cold PBS. Cells were scraped into 1ml of fresh PBS, the cell suspension transferred into 1.5ml reaction tubes and centrifuged at 2700g for 1 min. The supernatant was carefully removed and the cells were resuspended in 100ul of ice cold TK lysis buffer (20mM Tris HCl, pH 7.5, 2mM $MgCl_2$, 10mM NaCl, 6.5mM 2-mercaptoethanol, 0.5% v/v NP40) by vortexing and the tubes then placed on ice for 5 min. Samples were centrifuged at 11,600g for 2 min, and the supernatant was transferred to fresh tubes and maintained on ice or stored at -70^{o}C.

3.5.6. Thymidine Kinase Assays of Transfected Cells

Samples of cell extracts were assayed for TK activity in a reaction mixture containing 10mM Na_2HPO_4/NaH_2PO_4 pH6.0, 10mM $MgCl_2$, 5mM ATP, 0.1mMTTP and 100uCi/ml [^{3}H]-thymidine in 50ul at 30^{o}C. In most experiments a 5ul sample of cell extract and a 30min incubation period were used. The reaction was terminated by the addition of thymidine to 33uM and heating at 90^{o}C for 4 min. The tubes were then cooled on ice for 5 min, centrifuged at 11,600g for 2 min and 50ul of the supernatant spotted onto a DE81 filter paper disc.

The discs were washed three times in a solution of 4mM ammonium formate (pH4.0) and 10mM thymidine at 37°C and twice in absolute alcohol. Following drying under a heat lamp the discs were placed in vials and 5ml of scintillation fluid added. Incorporation of [^{3}H]-thymidine was determined by scintillation counting and used as a measure of TK activity.

3.5.7. Beta-galactosidase (B-gal) Assays of Transfected Cells.

Extracts prepared for use in CAT assays and TK assays were also used to assay B-gal activity. 25ul of extract was added to 175ul of Z buffer (60mM Na_2HPO_4, 4mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 20mM 2-mercaptoethanol, adjusted to pH 7.0) along with 40ul of O-nitrophenyl-B-D-galactopyranoside (4mg/ml) and incubated at 30°C for 30min to 4hr. Reactions were stopped by the addition of 100ul of 1M Na_2HCO_3, and the absorbance of the solution measured at 420nm.

3.6. Expression of VZV Proteins in E.coli.

3.6.1. Preparation of Extracts for TK Assays

A 2ml volume of L.Broth was inoculated with a single bacterial colony from an agar plate or 100ul of the appropriate glyoerol stock. After growth in a shaking incubator at 37 C for 2hr isopropyl-B.D-galactoside (IPTG) was added to a final concentration of 1mM and the incubation continued for a further 2-3hr. Bacteria were pelleted by centrifugation at 2,900g for 1min, and, after the supernatant had been discarded, resuspended in 100ul of TK lysis buffer with lysozyme added to a final concentration of 500ul/ml. This solution was incubated on ice for 10 min prior to freezing and thawing (-70°C to 37°C) three times. Centrifugation at 11,600g for 10 min resulted in a clarified supernatant which could be stored at -70°C.

3.6.2. Assays for TK Activity in E.coli extracts.

Two buffers were used in attempts to detect TK activity in lysates of bacterial cells. The first was that

previously optimised for the estimation of HSV TK activity (see section 3.5.6.). The second was one described for the analysis of VZV TK activity (50mM Tris HCl (pH7.5 or pH 8.0), 5mM [^{3}H]-thymidine, 2mM ATP, 2mM $MgCl_2$, 5mM 2-mercaptoethanol, with or without 5mM TTP) (Doberson et al., 1976; Shiraki et al., 1985). To both of these buffers was added 10ul of cell extract to make a total reaction volume of 50ul which was incubated at 30°C for 1hr. The rest of the assay was carried out exactly as described in section 3.5.6.

3.6.3. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE).

A 50ml volume of gel mix was prepared containing the required amount of 30% acrylamide / 3% DATD to give a final concentration of between 8% and 12% acrylamide in 1xGB (315mM Tris HCl, pH8.9, 0.1% SDS). Polymerisation was initiated by the addition of 0.5ml 10% w/v APS and 40ul TEMED, and the mixture was poured into a vertical gel mould (170mm/130mm/1.5mm). While still liquid the acrylamide was overlaid with 2ml GB and allowed to polymerise for 30min.

24ml stacking gel solution was prepared, containing 5% acrylamide, 0.5% DATD, 1xSGB (125mM Tris HCl, pH 6.7, 0.1% SDS), 0.5ml of 10% APS and 35ul of TEMED. The GB was removed from the surface of the running gel and, after washing, the mould was filled with the stacking gel solution and a suitable teflon comb inserted.

Samples were prepared by boiling for 5min in a denaturing buffer of final concentration 50mM Tris HCl (pH 6.7), 0.4% SDS, 10% glycerol, 5% 2-mercaptoethanol. Gels were electrophoresed for 3-4hr in a buffer containing 50mM Tris, 65mM glycine and 0.1% SDS and either fixed by soaking overnight in 25% methanol, 6% acetic acid prior to drying and autoradiography or stained in Coommassie Brilliant Blue for 1hr and destained in 25% methanol/6% acetic acid for 2-3 days.

3.7. DNA Binding Assays

3.7.1. Nuclear Extracts.

HeLa cell nuclear extracts were prepared as described by Preston et al. (1988), a method slightly adapted from that of Dingnam et al. (1981). Cells were scraped from 90mm diameter Petri dishes and centrifuged at 1500g for 5min at 4°C. Cell lysis was performed by resuspending the cells in 2 packed volumes of Buffer A (10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT and 0.5mM phenylmethylsulphonyl fluoride [PMSF]) containing 0.5% NP40, with intermittent mixing at 4°C for 10min. Nuclei were pelleted by centrifugation at 13,200g for 15min at 2°C. The supernatant was carefully discarded and the nuclei lysed by resuspending the pellet in 3ml of Buffer C (20mM HEPES ph7.9, 25% v/v glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF and 0.5mM DTT) per 10^9 cells, with intermittent mixing at 4°C for 30min. The suspension was centrifuged at 13,200g for 15min at 2°C and the supernatant (nuclear extract) collected and stored in small samples at -70°C.

3.7.2. Extraction of Virus Proteins for Gel Retardation Assays.

Cell released virus particles, ($1x10^{10}$), prepared as described in section 3.8.3. were concentrated by centrifugation at 25,000g for 2hr, resuspended in PBS/0.5% calf serum, and centrifuged through a cushion of 10% w/v sucrose in 10mm PBS at 25,000g for 2hr. The pellet was resuspended in 10mM Tris HCl, 50mM NaCl, 1mM EDTA (pH 7.5), NP40 added to a final concentration of 0.03%, and the samples kept on ice for 1hr. The extract was centrifuged at 50,000g for 1hr and the supernatant stored at -70°C.

Before the samples were analysed in gel retardation assays protein level comparisons were made by SDS-PAGE and Coommassie Brilliant Blue staining.

3.7.3. In Vitro Transcription of pGEMTIF and pGEMVZV and Translation of RNA Products.

The plasmids pGEMTIF and pGEMVZV A and B were cleaved with EcoRI before carrying out the transcription reaction. In vitro transcription was performed using the Riboprobe

system (Promega Biotech) following the manufacturers protocol and incubating 1 ug of plasmid DNA, 0.5 mM G(5')ppp(5')G (Pharmacia) and 0.4 mM of each nucleoside triphosphate in a total volume of 25ul at 37°C for 1hr.

In vitro translation was carried out by addition of 2.5ul of transcription mixture to 20ul of rabbit reticulocyte lysate (Amersham). Duplicate samples were incubated for 90 min at 30°C either in the presence or in the absence of 30uCi [^{35}S]methionine (sp. act.>800Ci/mmol) in a 25ul reaction mixture. Non-radioactive samples were stored at -70°C for use in gel retardation assays. The radiolabelled translational mixture was processed for SDS-PAGE as described in section 3.6.3.

3.7.4. 3'-end Labelling of Probes.

10ug of plasmid DNA was digested with appropriate restriction enzymes and purified by phenol/chloroform extraction and ethanol precipitation. The DNA fragments were then labelled in T4 polymerase buffer (33mM Tris acetate pH7.9, 66mM sodium acetate, 10mM magnesium acetate) containing 10mM DTT, 0.1% BSA, 0.5mCi/ml α-[^{32}P]-dATP, 0.5mCi/ml α-[^{32}P]-dTTP, 100uM dGTP, 100uM dCTP and 1 unit of T4 polymerase in a total volume of 20ul for 1hr at 37°C.

3.7.5. Gel Retardation Reactions.

Formation and analysis of protein-DNA complexes was performed as described by Preston *et al.* (1988). DNA fragments were end-labelled as described in section 3.7.4. and extracted from a polyacrylamide gel as described in section 3.4.3. Reaction mixtures contained the following components: 10mM HEPES pH 7·9, 0.6mM dithiothreitol, 2·3mM $MgCl_2$, 85mM NaCl, 0·1mg/ml bovine serum albumin, 4ug poly(dI).poly(dC), 0·2ng [^{32}P]-end labelled fragment (containing approximately 10,000 dpm), 5ug of mock-infected HeLa cell nuclear extract and, where appropriate, 0.3ug-1.0ug of virion extract, Vmw65 or orf10 synthesised *in vitro*. Incubation was at 25°C for 30min, and reaction mixtures were loaded directly onto a 3·5% polyacrylamide gel. After electrophoresis for 3·5hr at 160V, the gel was

dried and exposed for autoradiography. If assays involved the use of competitor oligonucleotides, a 100-fold molar excess was incubated with the rest of the components of the reaction for 15 min prior to the addition of the radiolabelled fragment.

3.7.6. DNAaseI Protection Assays

Gel retardation reaction mixtures of 60ul were assembled, containing approximately 10^5cpm of DNA fragment labelled uniquely at one 3' end. After incubation at 25°C for 30min, 7ul of 150ug/ml DNAaseI was added, and incubation continued at 25°C for 3min. EDTA was added to a final concentration of 10mM, and samples were loaded onto a 3.5% polyacrylamide gel. After electrophoresis, the gel was sliced, and the peaks representing IEC and free DNA were identified by Cerenkov counting of the slices. Complexed and free DNA were recovered by electroelution, extracted once with phenol/chloroform, once with chloroform, and precipitated with ethanol. DNA fragments were analysed by electrophoresis on a 10% polyacrylamide gel containing 0.5xTBE and 7M urea. Markers were obtained by using G and G+A sequencing reactions as described in section 3.3.6.

3.8. Constuction and Purification of HSV mutants.

3.8.1. Co-transfection of Virus DNA and Plasmid DNA into Cells.

Plasmid DNA was linearised (within the vector sequences) and 0.5ug transfected with 0.5ug of virus DNA into cells as described in section 3.5.1., except thåt 2.0ug of calf thymus DNA was used as carrier and precipitates were gently mixed on addition of $CaCl_2$ and not vortexed. Transfected monolayers were incubated in ETC_{10} at 31°C for 3 days. The virus progeny were isolated by scraping into the growth medium which was transferred into a 5ml glass vial. Cells were then disrupted by sonication and the sonicate stored at -70°C.

3.8.2. Isolation of Virus Plaques.

A series of 10-fold dilutions in ETC_{10} were made from virus stocks prepared as describe in section 3.8.1. The medium was removed from confluent cell monolayers and 200ul of diluted virus added to each dish. Following virus absorption at 37°C for 1hr medium was carefully removed and the cells overlaid with 4ml of agar growth medium (Eagles medium containing 10% NBC, 10% TP and 0.5% Dif co Bacto Noble agar). Plates were incubated at 31°C for three days until virus plaques were visible and then overlaid with 2ml of agar growth medium containing 1.8ug of 5-Bromo-4-chloro-3-indolyl-B.D-galactoside (X-gal). Plates were then incubated for a further 16hr and inspected for plaques surrounded by the blue colour of metabolised X-gal. If the colour produced was not intense the plates were incubated overnight at 38.5°C. Single plaques were scraped up in approximately 50ul of agar using a micropipette, transferred to 200ul of ETC_{10}, sonicated and stored at -70°C.

3.8.3. Large Scale Preparation of Virus Stocks

Confluent BHK monolayers in 850cm^2 roller bottles were infected with 0.03 pfu of virus per cell in 50ml ETC_{10}. After incubation at 31°C for 3-4days, when extensive cpe had developed, cells were shaken into their medium and centrifuged at 1,000g for 15min at 4°C to produce a pellet containing infected cells and a supernatant containing cell released virus (CRV). The cell pellet was sonicated in 1ml of ETC_{10} to release the virus and the cell debris pelleted by centrifugation at 1,000g for 15min at 4°C. The supernatant designated cell associated virus (CAV) was stored in aliquots at -70°C. CRV was purified by centrifugation at 13,200g for 2hr at 4°C, the virus pellet was sonicated, resuspended in 10ml ETC_{10} and stored at -70°C in small aliquots.

3.8.4. Large Scale Preparation of Viral DNA

CRV was prepared as described (section 3.8.3.), however after centrifugation at 13,200g for 2hr at 4°C the virus

pellet was resuspended in 20mM Tris HCl (pH 7.5), 100mM NaCl, 2mM EDTA and 0.2% SDS containing 0.25mg/ml proteinase K, and incubated at 37°C overnight with gentle agitation. DNA was extracted three times with phenol/chloroform and once with chloroform, precipitated in two volumes of ethanol, dried and dissolved in 0.4ml of water.

3.8.5. Small Scale Preparation of Virus-Infected Cell DNA.

Confluent cell monolayers on 50mm diameter petri dishes were infected at 1 pfu/cell. Following virus absorption at 37°C 5ml of ETC_{10} was added and the plates incubated at 31°C for 18hr. The medium was decanted, and the cells were washed with 5ml PBS, scraped into 1ml PBS and pipetted into a 1.5ml vial. Vials were centrifuged at 2700g for 3min, the supernatant discarded and 0.4ml of TK lysis buffer added. Samples were then incubated on ice for 5min and centrifuged at 11,600g for 2min. The supernatants were collected and EDTA, SDS and NaCl added to final concentrations of 5mM, 0.2% and 0.1M, respectively. DNA was purified by two phenol/chloroform extractions, one chloroform extraction and precipitation in 2 volumes of ethanol. Vials were centrifuged at 11,600g for 10min and the pellets washed in ethanol, dried under vacuum and dissolved in 40ul of water.

3.9. Southern Blot Analysis of Virus DNA.

3.9.1. Internal Labelling of Probes by Nick Translation.

DNA was labelled with ^{32}P as described by Rigby et al., (1977). 1.0ug of plasmid DNA was incubated in a reaction mix containing 40mM dATP, 40mM dTTP, 2.5ul of 10xNTB (5M Tris HCl, pH7.5, 1M $MgCl_2$, 1mM DTT and 5mg/ml BSA) and $2x10^{-4}$ug DNAaseI at RT for 3min. The mix was placed on ice and diluted to 25ul by the addition of 30uCi of both α-[^{32}P]-dCTP and α-[^{32}P]-dGTP, 3 units of E.coli DNA polymerase I plus distilled water. ^{32}P labelled DNA was then separated from unincorporated triphosphates by centrifugation (1500g for 4min) through a 1ml Sephadex (fine) G50 column that had been tightly packed by

centrifugation (1500g, 4min) in a plastic syringe.

3.9.2. Transfer of DNA to Nitrocellulose.

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

3.9.3. DNA/DNA Hybridisation.

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

The prehybridisation mixture was removed and replaced with the denatured probe in a mix of the same final composition as the prehybridisation solution. The nitrocellulose filter was shaken overnight at 65^{o}C then washed in 10xDenhardts/4xSSC for 30min at RT, followed by three washes in 0.3xSSC, 0.1% SDS at 65^{o}C. It was air dried and autoradiographed.

3.10. Analysis of Viral Proteins

3.10.1. Radiolabelling of Viral IE Polypeptides.

An incubation of 38.5°C was used throughout. Cell monolayers in 30 mm diameter Petri dishes were infected with a moi of 1 pfu/cell and the virus incubated for 1hr to allow absorption. The cells were then overlayed with 2ml an ETC_{10} pre heated to 38.5°C and incubated for a further 4hr. The medium was then removed from the dishes and the cells washed with PBS. 0.3ml of PBS containing 100uCi/ml [^{35}S]-methionine was added and the monolayers incubated for a further 1hr before being harvested.

3.10.2. Preparation of Protein Samples for SDS-PAGE.

The medium was removed from each dish and the monolayers washed with ice cold PBS. The cells were then rapidly washed with ice cold TE (10mM Tris HCl, pH7.5, 1mM EDTA), 0.3ml TE was added and the monolayers incubated at 4°C for 10min. The cells were transferred to a 1.5ml vial and denatured by boiling for 20min in "boiling mix". Samples of 25ul were analysed by SDS-PAGE (Section 3.6.3.)

4. RESULTS

4.1. Mapping the 5' terminus of the VZV major IE RNA.

To analyse the transcriptional regulation of VZV orf62, 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 29bp of the N-terminal coding sequence of orf62 (Figure 4.1.). This particular sequence was chosen to ensure hybridisation within the mRNA and also to minimise the amount of coding sequences transcribed by the reverse transcriptase. Figure 4.1. shows that an extension product of approximately 105 bases was produced from three separate VZV-infected cell RNA preparations but not from mock-infected cell RNA. This indicates that the mRNA 5' terminus is about 105 bases from the 5' end of the primer and thus that the length of the 5' noncoding region is 71 bases as the 5' end of the oligonucleotide hybridises 34 bases 3' to the proposed initiator ATG. No other higher molecular weight products were present on the autoradiograph, suggesting that the 105 base band represents the true 5' terminus rather than a "strong stop" for reverse transcriptase. To confirm this resultwith increased precision, and to exclude the existence of an intron within the 5' noncoding region, nuclease S1 analysis was carried out using a radiolabelled probe that spanned the 5'terminus predicted by primer extension. As shown in Figure 4.2., a cluster of protected DNA bands of 44, 45 and 46 bases were observed when VZV-infected cell RNA, (lane 6), but not mock-infected cell RNA, (lane 5) was used. These data place the start site of the mRNA at 73, 74, and 75 bases upstream of the proposed ATG and as such basically confirm the results gained from the primer extension assay. The resolution of the gel and the precision of the marker tracks allow a more accurate definition of the mRNA start site by nuclease S1 analysis, and as such the mRNA start site was assigned to the C residue shown in figure 4.1., 74 bases upstream of the proposed ATG. These assays together exclude

Figure 4.1. DNA sequences in the upstream regulatory region of VZV orf 62.

Restriction endonuclease cleavage sites relevant to the results are named and underlined. The mRNA start site is by convention designated +1, a 'TATA' box homology TTTTAA, is present at position -25 to -30. A 13bp sequence centered at -255 contains both octamer and TAATGARAT motifs and a simple octamer motif in inverse orientation is present at nucleotides -346 to -339. In addition, the CCAAT box ATF/CRE, and PEA-2 motifs centred at -117, -73 and -50, respectively, are underlined and labelled. 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. The origin of DNA replication (residues -882 to -1140) is marked and labelled, as are the repeated sequences Reiteration R4 (residues -550 to -699).

A loose copy of this figure is provided at the back of the thesis.

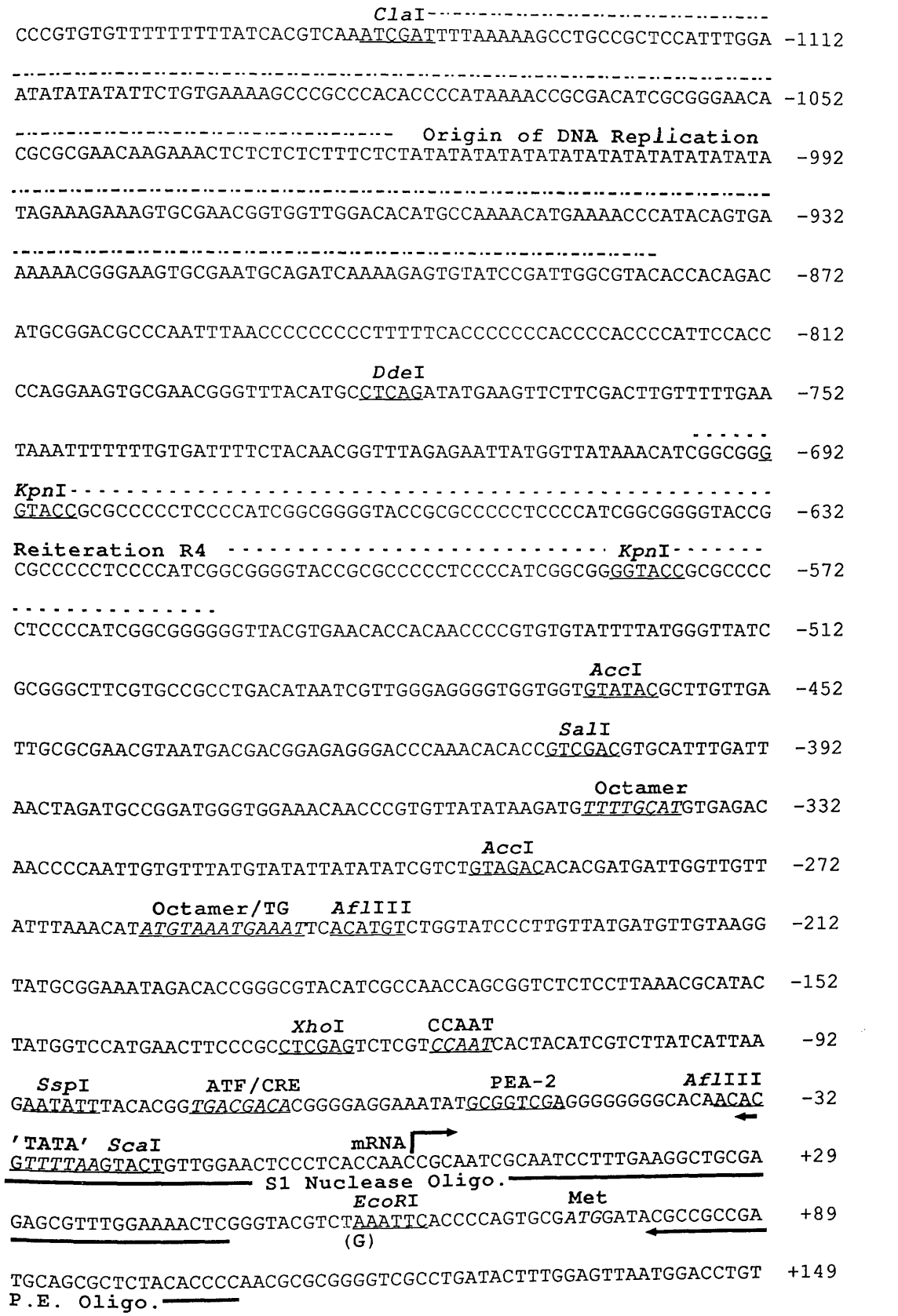
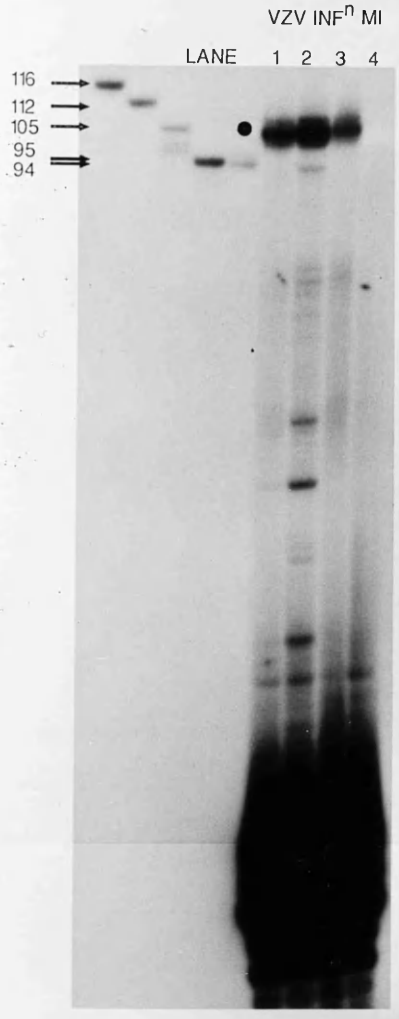

ClaI
CCCGTGTGTTTTTTTTTATCACGTCAAATCGATTTTAAAAAGCCTGCCGCTCCATTTGGA -1112
ATATATATATTCTGTGAAAAGCCCGCCCACACCCCATAAAACCGCGACATCGCGGGAACA -1052
Origin of DNA Replication
CGCGCGAACAAGAAACTCTCTCTCTTTCTCTATATATATATATATATATATATATATA -992
TAGAAAGAAAGTGCGAACGGTGGTTGGACACATGCCAAAACATGAAAACCCATACAGTGA -932
AAAAACGGGAAGTGCGAATGCAGATCAAAAGAGTGTATCCGATTGGCGTACACCACAGAC -872
ATGCGGACGCCCAATTTAACCCCCCCCCTTTTTCACCCCCCCACCCCACCCCATTCCACC -812
DdeI
CCAGGAAGTGCGAACGGGTTTACATGCCTCTCAGATATGAAGTTCTTCGACTTGTTTTTGAA -752
TAAATTTTTTTGTGATTTTCTACAACGGTTTAGAGAATTATGGTTATAAACATCGGCGGG -692
KpnI
GTACCGCGCCCCCTCCCCATCGGCGGGGTACCGCGCCCCCTCCCCATCGGCGGGGTACCG -632
Reiteration R4
KpnI
CGCCCCCTCCCCATCGGCGGGGTACCGCGCCCCCTCCCCATCGGCGGGGTACCGCGCCCC -572
CTCCCCATCGGCGGGGGGTTACGTGAACACCACAACCCCGTGTGTATTTTATGGGTTATC -512
AccI
GCGGGCTTCGTGCCGCCTGACATAATCGTTGGGAGGGGTGGTGGTGTATACGCTTGTTGA -452
SalI
TTGCGCGAACGTAATGACGACGGAGAGGGACCCAAACACACCGTCGACGTGCATTTGATT -392
Octamer
AACTAGATGCCGGATGGGTGGAAACAACCCGTGTTATATAAGATGTTTTGCATGTGAGAC -332
AccI
AACCCCAATTGTGTTTATGTATATTATATATCGTCTGTAGACACACGATGATTGGTTGTT -272
Octamer/TG
AflIII
ATTTAAACATATGTAAATGAAATTCACATGTCTGGTATCCCTTGTTATGATGTTGTAAGG -212
TATGCGGAAATAGACACCGGGCGTACATCGCCAACCAGCGGTCTCTCCTTAAACGCATAC -152
XhoI
CCAAT
TATGGTCCATGAACTTCCCGCCTCGAGTCTCGTCCAATCACTACATCGTCTTATCATTAA -92
SspI
ATF/CRE
PEA-2
AflIII
GAATATTTACACGGTGACGACACGGGGAGGAAATATGCGGTCGAGGGGGGGGCACAACAC -32
'TATA'
ScaI
mRNA
GTTTTAAGTACTGTTGGAACTCCCTCACCAACCGCAATCGCAATCCTTTGAAGGCTGCGA +29
S1 Nuclease Oligo.
EcoRI
Met
GAGCGTTTGGAAAACTCGGGTACGTCTAAATTCACCCCAGTGCGATGGATACGCCGCCGA +89
(G)
TGCAGCGCTCTACACCCCAACGCGCGGGGTCGCCTGATACTTTGGAGTTAATGGACCTGT +149
P.E. Oligo.

Figure 4.2. Mapping the 5' end of the mRNA.

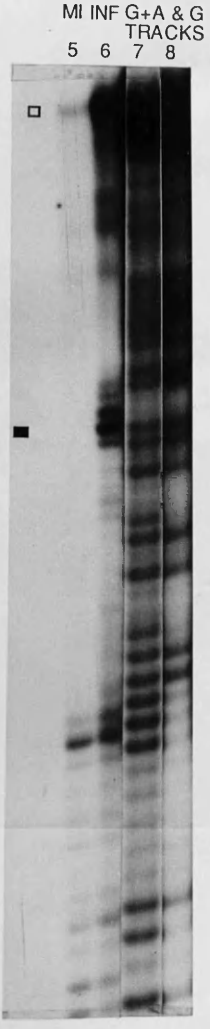
Primer extension analysis was performed on three RNA samples prepared from different batches of VZV infected HFL cells (lanes 1, 2 and 3) and from mock infected HFL cells (lane 4). Markers of the indicated sizes (bases) were run in parallel. Extended product is labelled (●).

Nuclease S1 analysis was performed on RNA prepared from VZV infected HFL cells (lane 6) or mock infected cells (lane 5). G and G+A tracks 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.

PRIMER EXTENSION



S1 NUCLEASE MAPPING



The formal possibility remains that these findings could be explained by the presence of two introns. One of these would represent some or all of the residues between +80 and +46 in figure 4.1. The downstream end of the other intron would have to be precisely at the residue designated +1 in figure 4.1., this intron could be of any size and thus could terminate upstream of the S1 nuclease oligonucleotide. This possibility could be excluded by nuclease S1 analysis using a probe spanning residues +80 to +46.

the possibility that splicing occurs within the the 5' untranslated region. The presence of an intron in the 5' untranslated region would have resulted in a discrepancy between results of the primer extension and the S1 nuclease analyses, whereby the 5' terminus predicted by primer extension would have mapped downstream of that predicted by S1 nuclease analysis by the length of the intron. (see over)

The presence of more undigested probe in figure 4.2. lane 6 (infected cell RNA) than lane 5 (M.I. cell RNA) was noted. This finding suggested the possibility of protection of the probe by longer transcripts. This was not a consistent finding since in other experiments at least as much undigested probe was present in mock infected tracks. Variations in the amount of undigested probe within an experiment could result from differences in the amount of viral DNA present in the RNA preparations. The presence of viral DNA is a particular problem with VZV infections as coordinate infections are impractical because of difficul ties in isolating cell free virus at high titres (Section 1.2.2.). Thus at 24 hours, when RNA was extracted, considerable amounts of viral DNA would have been present, given that virus DNA replication has been detected only 6 hours after infection of cell monolayers (Gelb, 1985). It is impossible to be certain as to the amount of viral DNA that was present in the RNA preparations used. The possibility of nonspecific hybridisation to other viral transcripts also cannot be excluded. The formal possibility also exists that protection of the entire probe from S1 nuclease digestion was by a bona fide viral transcript. If so it was not detected by primer extension analysis and thus must either have terminated in the 32 bases between the 5' ends of the oligonucleotides employed for S1 nuclease and primer extension analysis or continued so far upstream of the primer extension oligonucleotide that its 5' end was not detected by primer extension analysis.

Inspection of the sequence (Figure 4.1.) reveals the element TTTTAA centred at 28 bases 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 (Section 1.5.1.).

4.2. Functional Studies

4.2.1. Plasmid Construction

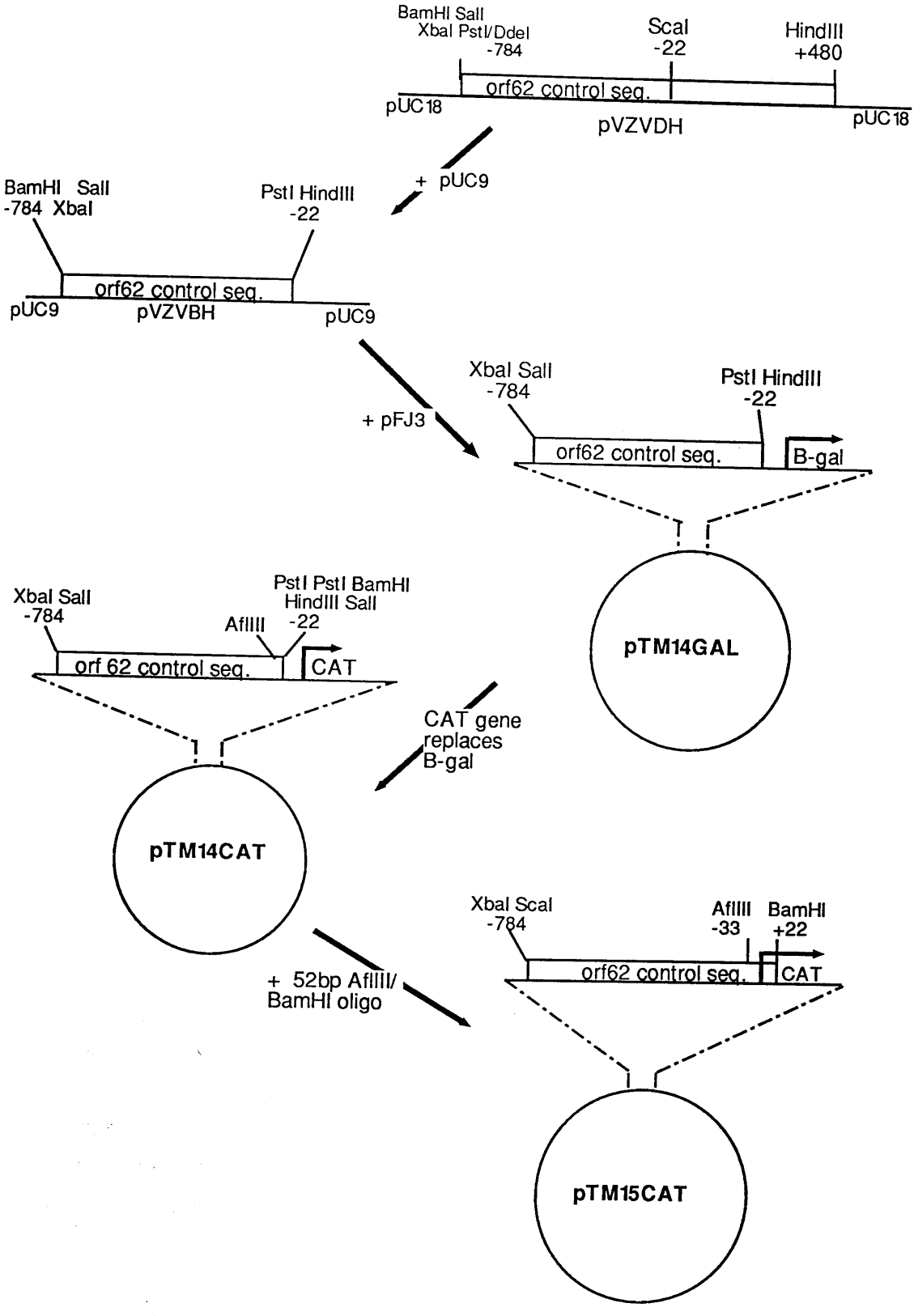
Having defined the location of the mRNA start site, attempts were made to characterise functional aspects of the regulatory sequences. In order to achieve this plasmids were constructed using CAT and B-galactosidase as reporter genes driven by sequences present 5' to orf62. These can be considered in two groups, both derived from the VZV genomic clone pVZVSstf, (kindly provided by Dr. A. Davison). Plasmid pVZVSstf contains the terminal SstI fragment from the short repeat region of VZV cloned into the PstI site of pAT153 by GC tailing, creating a PstI site at the genome terminus.

The first group of plasmids (Figure 4.3.) was produced using a 1265bp DdeI-HindIII fragment of pVZVSstf which was inserted into pUC18 between the PstI site, blunt ended using T4 DNA polymerase, and the HindIII site, creating pVZVDH. A BamHI-ScaI fragment (residues -781 to -22) was excised from this plasmid and inserted into the pUC9 polylinker using the BamHI and HincII sites, creating pVZVBH. Finally a XbaI-HindIII fragment was excised and inserted between the XbaI and HindIII sites of pFJ3 which contains the E.coli B-galactosidase gene with SV40 early gene termination signals (kindly provided by Dr. F. Rixon) to form the plasmid pTM14gal. An equivalent CAT construct (pTM14cat) was produced by replacing the HindIII-BamHI fragment of pTM14gal, which contains the B-galactosidase gene, with the HindIII-BglII fragment of pBLW2, which contains the CAT gene.

The plasmid pTM14cat was then modified by the insertion of a 52bp double stranded oligonucleotide encoding VZV sequences between -33 and +20 (Appendix 3; figure 4.2). This oligonucleotide was designed to have 5' overhanging ends complementary to those produced by the restriction enzymes AflIII and BamHI. A partial digest of pTM14cat was carried out using AflIII and the product cleaved to completion with BamHI. The appropriate fragment was

Figure 4.3. Production of Plasmids pTM14gal, pTM14cat and pTM15cat.

VZV sequences are represented by an open box and labelled. The messenger RNA start site is marked along with the reporter gene used. Relevant restriction endonuclease cleavage sites are labelled. pUC18 was digested with PstI and HindIII, the PstI site was blunt-ended and the VZV fragment inserted to form pVZVDH. Digestion with BamHI and HincII allowed the introduction of the fragment containing the putative orf62 regulatory sequences into pUC9. The HindIII and XbaI sites in pFJ3 allowed the replacement of SV40 control sequences with those of orf62 to form pTM14gal. The CAT gene that replaced the B-gal gene to form pTM14cat, were contained within a HindIII-BglII fragment. The sequence of the double stranded oligonucleotide (oligonucleotide 3) is provided in Appendix 3, and is identical to the sequence between -30 and +22.


BamHI SalI
XbaI PstI/DdeI
-784
ScaI
-22
HindIII
+480
orf62 control seq.
pUC18
pVZVDH
pUC18
+ pUC9
BamHI SalI
-784 XbaI
PstI HindIII
-22
orf62 control seq.
pUC9
pVZVBH
pUC9
+ pFJ3
XbaI SalI
-784
PstI HindIII
-22
orf62 control seq.
B-gal
pTM14GAL
XbaI SalI
-784
PstI PstI BamHI
HindIII SalI
AflIII
-22
orf 62 control seq.
CAT
CAT gene
replaces
B-gal
pTM14CAT
XbaI ScaI
-784
AflIII
-33
BamHI
+22
orf62 control seq.
CAT
+ 52bp AflIII/
BamHI oligo
pTM15CAT

purified and the oligonucleotide inserted. This resulted in a plasmid in which the polylinker sequences, present in pTM14cat, were replaced by VZV sequences, including the mRNA start site. The plasmid was named pTM15cat.

A completely separate construction, p140cat, encoding the regulatory sequences and the CAT gene was, constructed by G. Disney, again using pVZVSstf (McKee et al., in press). This plasmid was constructed from p140 which contains VZV sequences from the ClaI site at -1146 to the genomic terminus PstI site of pVZVSstf excised and inserted between the AccI and PstI sites of a pUC9 plasmid from which the EcoRI site had been removed by cutting and filling in. Plasmid p140ET is a derivative of p140 in which an EcoRI site was created by oligonucleotide mutagenesis of nucleotide +55 relative to the VZV orf 62 mRNA start site, changing the sequence AAATTC to GAATTC (figure 4.1.). The new EcoRI site in p140ET was converted to a BglII site (p140BT) by cleaving with EcoRI, filling in and inserting an oligonucleotide linker (GAGATCTC). Using this novel BglII site and a SmaI site in vector sequences immediately 5' of the VZV insert, the orf62 region from -1146 to +55 of p140BT was cloned between the SmaI and BamHI sites of pCAT to give p140cat (Figures 4.4. and 4.5.).

Sequential 5' deletions of p140cat, pTM14cat and pTM14gal were made using the SalI site at -410 and the XhoI site at -131 forming p140Δ410cat, pTM14Δ410cat and pTM14Δ410gal and p140Δ131cat, pTM14Δ131cat and pTM14Δ131gal respectively (Figure 4.5.). In pTM14cat and pTM14gal the SalI and XhoI sites were digested and ligated to a SalI site present immediately 5' to the VZV sequences. In p140cat they were filled in using T4 polymerase and ligated to a SmaI site again found immediately 5' to the VZV sequences.

The reasons for the construction of these different vectors encoding different reporter genes under the control of orf62 regulatory signals are related to the twin problems encountered throughout this part of the project, namely, the low level of transcriptional activity induced by the control sequences of orf62 and the inconsistent nature of short term transfection assays (Everett et al., 1989).

4.2.2. Analysis of Control Region Deletions

Figure 4.4. Parental Plasmids used in Transfection Assays.

Schematic representations of plasmids are presented. VZV sequences are represented by heavy lines. The open box marked R4 represents Reiteration R4 and the shaded box marked Ori represents an origin of DNA replication. The octamer motif is marked o, the combined octamer/TAATGARAT motif is marked o+ and the promoter fragment, ****. the position of both ends of the VZV sequences incorporated in the plasmid are given and the approximate position of the mRNA start site is marked ⌐→. The positions of the various cis-acting sequences are approximate.

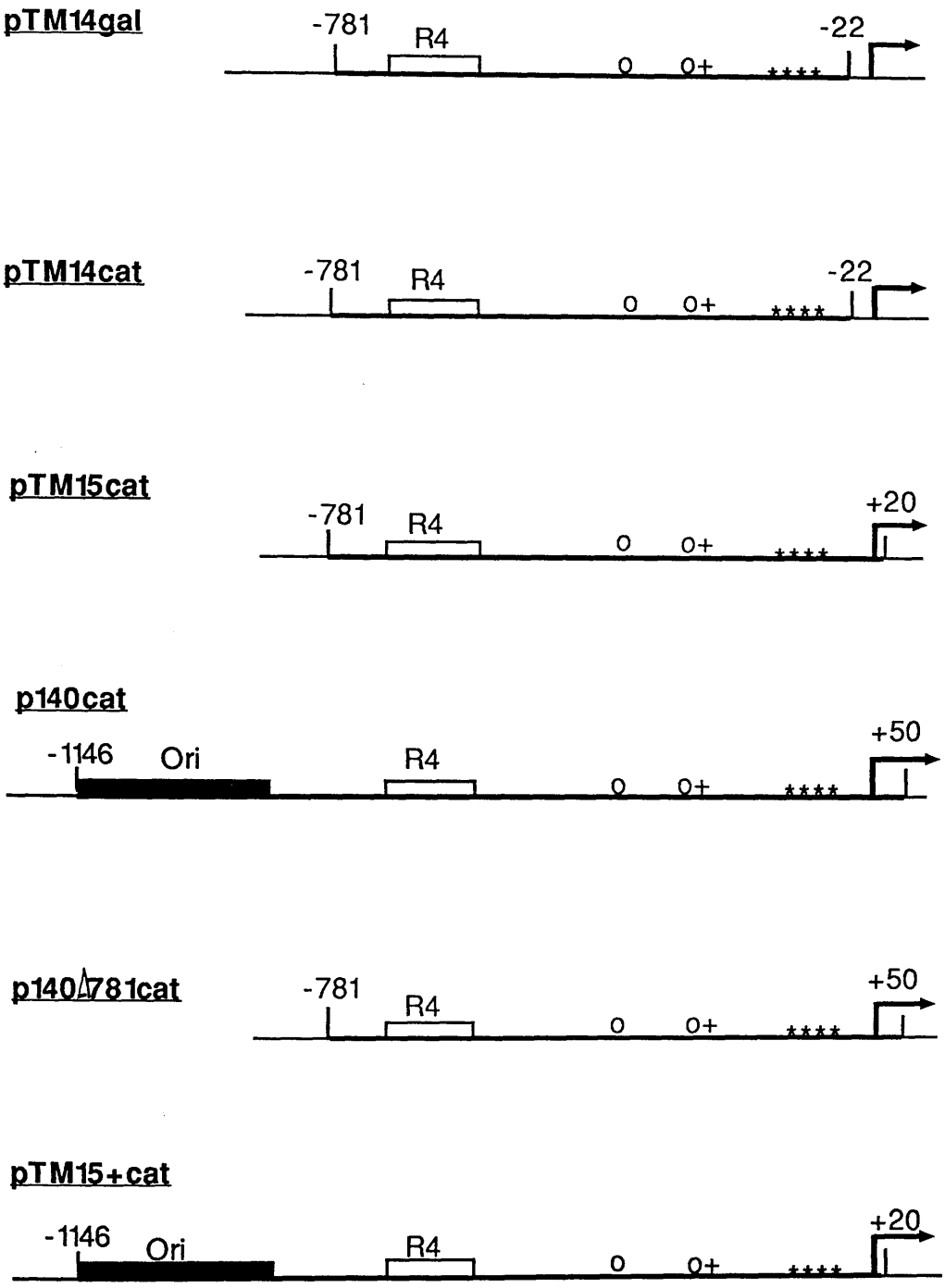
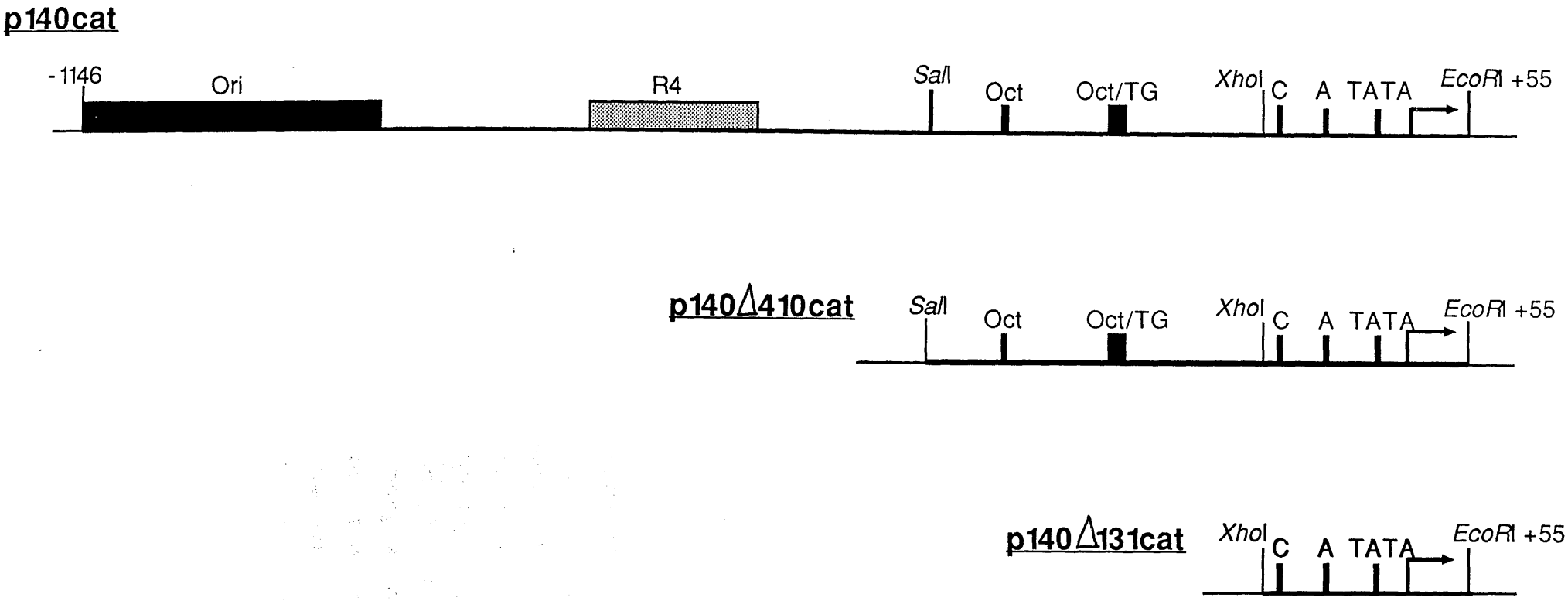

pTM14gal
-781
R4
-22
o
o+
****
pTM14cat
-781
R4
-22
o
o+
****
pTM15cat
-781
R4
+20
o
o+
****
p140cat
-1146
Ori
R4
+50
o
o+
****
p140Δ781cat
-781
R4
+50
o
o+
****
pTM15+cat
-1146
Ori
R4
+20
o
o+
****

Figure 4.5. Deletions of VZV coding sequences derived from pl40cat

VZV coding sequences are marked by a heavy line, plasmid sequences by a faint line. Boxes marked Ori and R4 represent an origin of DNA replication and R4 Reiterations respectively. Relevant restriction endonuclease cleavage sites are marked, and the mRNA start site is marked thus ⌐. Cis-acting motifs present are annotated as follows.

Oct	-	octamer	TTTTGCAT
Oct/TG	-	octamer/TAATGARAT	ATGTAAATGAAAT
C	-	CCAAT box	CCAAT
A	-	ATF/CRE site	TGACGACA
TATA	-	'TATA'	TTTTAA

Equivalent deletions were produced from pTM14gal and pTM14cat.

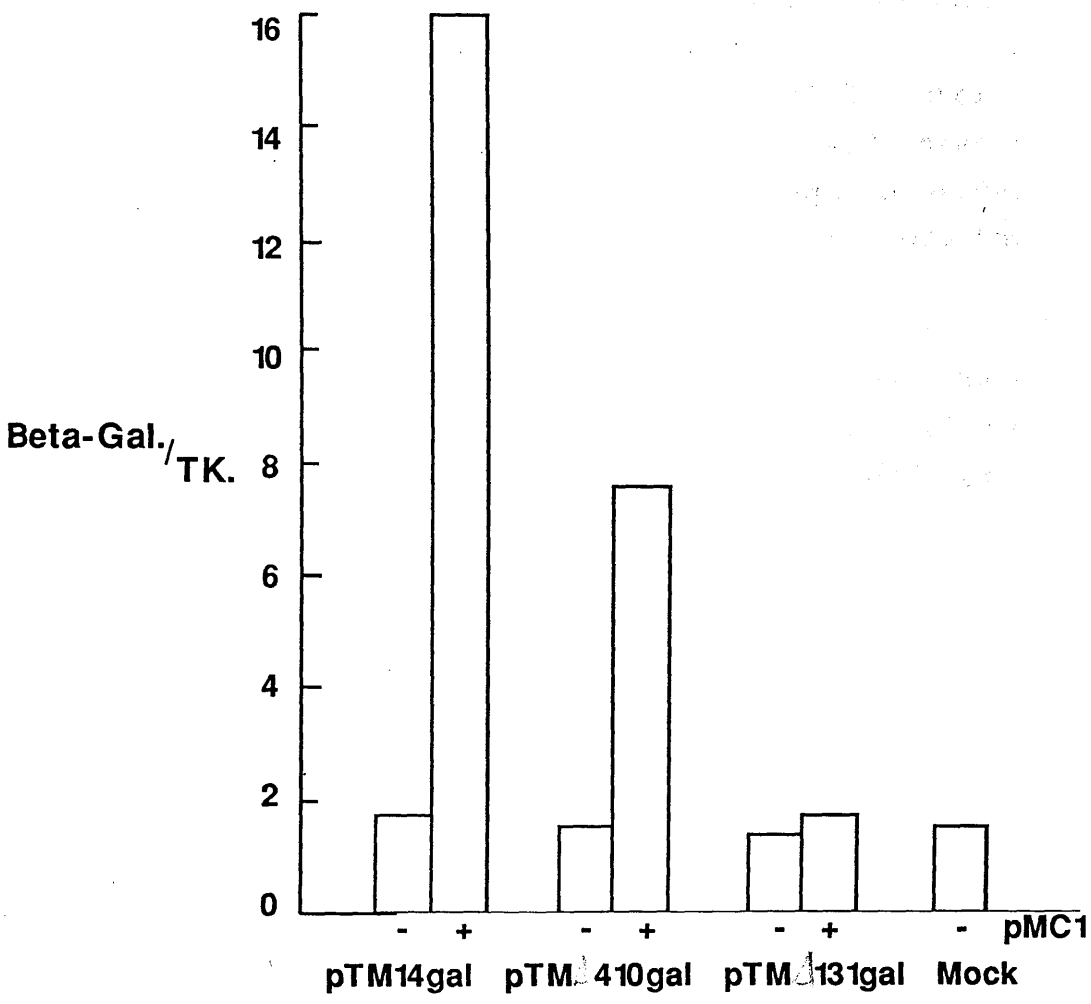

p140cat
-1146
Ori
R4
SalI
Oct
Oct/TG
XhoI
C
A
TATA
EcoRI +55
p140Δ410cat
SalI
Oct
Oct/TG
XhoI
C
A
TATA
EcoRI +55
p140Δ131cat
XhoI
C
A
TATA
EcoRI +55

Short term transfection assays described in the following sections were all performed with an internal control. In the case of the B-galactosidase assays the internal control was either pFS8, a plasmid that expresses the HSV-1 TK gene under the control of SV40 E gene regulatory sequences, or pSV2CAT, which contains the CAT gene under the control of the same regulatory sequences. VZV derived plasmids encoding the CAT gene were co-transfected with pFJ3, which expresses the B-galactosidase gene under the control of SV40 regulatory sequences. The rationale behind these procedures was that assays of the control plasmid expression could be used to normalise the test plasmid assays, thus reducing the effect of variability between transfections. In the case of CAT assays this was achieved by altering the amount of extract present in the assays while for B-gal assays a numeric conversion was used.

Results of an early experiment in which pTM14gal, pTM14Δ410gal and pTM14Δ131gal were transfected into BHK cells with or without pMC1, which encodes HSV-1 Vmw65, are shown in figure 4.6. In the absence of pMC1 no B-galactosidase activity was detected; however, co-transfection of this plasmid resulted in detectable activity from cells transfected with pTM14gal or pTM14Δ410gal but not pTM14Δ131gal. The results summarise the basic findings derived from analysis of the regulatory sequences of orf62 which were later confirmed using other constructs. They are as follows; first, a low level of baseline expression, which could not be detected above background in this experiment, was generated by cis-acting sequences of orf62 and cellular factors; second, this level of activity was not increased significantly, if at all, by the presence of sequences 5' to the XhoI site at -131, at least not enough to become detectable above background in this experiment; third, sequences 5' to -131 confer responsiveness to stimulation by Vmw65 resulting in a large relative increase in expression. The results gained from this experiment, however, were not reproduced in other experiments using pTM14gal because transfection efficiency, as measured by the expression of the internal control plasmid, was not sufficiently high to detect production of

Figure 4.6. An Early Transfection Experiment Using Plasmid pTM14gal and deletions.

Transfections were normalised using the plasmid pFS8 which encodes the HSV TK genes with SV40 control sequences. Results represent the average of two independent experiments. The result of the B-galactosidase assays were divided by the result of a TK assay of the same extract to give the normalised units presented on the ordinate. The reporter plasmids shown were co-transfected in the presence (+) or absence (-) of the plasmid pMC1. Total amounts of plasmid were kept constant with pUC18.


Beta-Gal./TK.
16
14
12
10
8
6
4
2
0
- + - + - + - pMC1
pTM14gal
pTM410gal
pTM131gal
Mock

B-galactosidase even in the presence of pMC1.

Obviously this situation, especially the inability to detect baseline expression, was unsatisfactory. It was known that eukaryotic cells produce endogenous B-galactosidase activity and it seemed possible that this background activity obscured the signal from transfected plasmids in the absence of pMC1. To address this question a construct, pTM14cat, was produced as described (section 4.1.2.) and transfected into BHK cells. No CAT activity could be detected in this experiment in the presence or absence of pMC1, despite detectable levels of control plasmid activity.

BHK cells, the standard cell type used for short term transfection assays, are not permissive for VZV infections. It was considered possible that the blockage to productive infection was at the level of IE transcription, thus explaining the low expression of pTM14gal and pTM14cat. Therefore both pTM14cat and pTM14gal were assayed in Vero cells, which are permissive for VZV infection. Again however, while low levels of activity, five fold above background, were detected in assays in which pTM14gal and pMC1 were present, unstimulated activity from pTM14gal could not be detected above background, and no CAT activity could be detected in cells transfected with pTM14cat in the presence or absence of pMC1.

In considering further the possible reasons for the low levels of expression from these plasmids, it was noted that neither pTM14gal nor pTM14cat contained the VZV orf62 mRNA initiation site. The plasmids had 81bp and 50bp respectively between the TATA box and the ATG of their reporter genes, sufficient for the normal initiation of transcription. However, it remained possible that other signals were present around the mRNA initiation site which normally contributed to increased gene expression. The plasmid pTM15cat (figure 4.4.) was constructed as described to investigate this possibility. Reporter gene expression from this plasmid was assayed in BHK cells but found to be not significantly higher than pTM14gal.

When the plasmid p140cat became available (section 4.2.1.), an experiment was conducted in BHK cells to compare

the activity of this plasmid with pTM14cat and pTM15cat. The result (Figure 4.7.), shows a high level of expression from p140cat compared with the activity obtained from cells transfected with pTM14cat and pTM15cat. Similar results were obtained in parallel experiments by G. Disney (unpublished observations). In view of these results, 5' end point deletions were made as described above (Figure 4.5.) and plasmids p140Δ410cat and p140Δ131cat compared with p140cat by transfection in both BHK and HeLa cells (Figure 4.8.). The data from a series of such experiments are given in table 4.1. They confirmed the results gained with pTM14gal in that deletion to -410 or -131 did not significantly reduce the expression of CAT in either cell type in the absence of pMC1. When pMC1 was included in transfection mixtures, expression of p140Δ410cat was stimulated on average 22-fold in BHK cells and 15-fold in HeLa cells and p140cat was stimulated 38 fold and 24 fold in the same cell types. The expression of p140Δ131cat was stimulated by 1.2 fold in BHK cells and by 2.25 fold in HeLa cells. In view of the much higher stimulations of other plasmids in both cell types it was considered that these stimulations were not significant. Therefore sequences that respond to Vmw65 are largely located between -410 and -131, but sequences that determine basal expression are located within the 131bp upstream of the mRNA 5' terminus.

To summarise this section, despite problems with transfection efficiency and an unexpectedly low activity of the orf62 promoter, positive results were obtained using B-galactosidase as a reporter in BHK cells (figure 4.5.) and Vero cells and CAT as reporter in both BHK cells and HeLa cells (figure 4.8., table 4.1.). The orf 62 promoter responds to stimulation by Vmw65, and sequences indispensible for this effect lie upstream of -130. Basal promoter activity is conferred by DNA sequences within the region -130 to +55.

4.2.3. Comparison of p140cat with other Herpesvirus IE Control Sequences

An experiment was undertaken to make a direct

Figure 4.7. Initial comparison of pl40cat, pTMl5cat and pTMl4cat.

As in all CAT assays, results were initially normalised using co-transfection with pFJ3, a plasmid that expresses B-galactosidase under the control of SV40 early control sequences. B-galactosidase results were used to adjust the amount of extract used in CAT assays. This experiment shows a comparison of pl40cat (lanes 1 and 2), pTMl5cat (lanes 3 and 4) and pTMl4cat (lanes 5 and 6) in the presence (+) and absence (-) of pMCl.

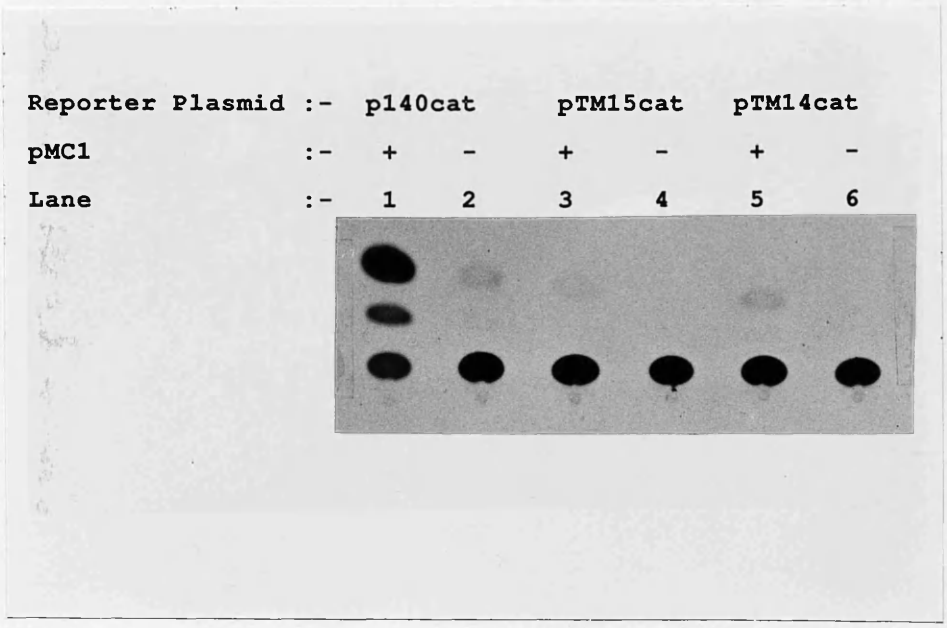

Reporter Plasmid :- p140cat pTM15cat pTM14cat
pMC1 :- + - + - + -
Lane :- 1 2 3 4 5 6

Figure 4.8. Activity of pl40CAT and Deletion Mutants in BHK and HeLa Cells.

To demonstrate the responsiveness of pl40cat and the deletion mutants derived from it to Vmw65, tracks marked + were cotransfected with pMC1 and those marked - with pUC18. Samples 10-16 were excised from the same autoradiograph and represent equal exposures.

Lanes 1-9 are the result of BHK cell transfections.
Lanes 10-16 are the result of HeLa cell transfections.

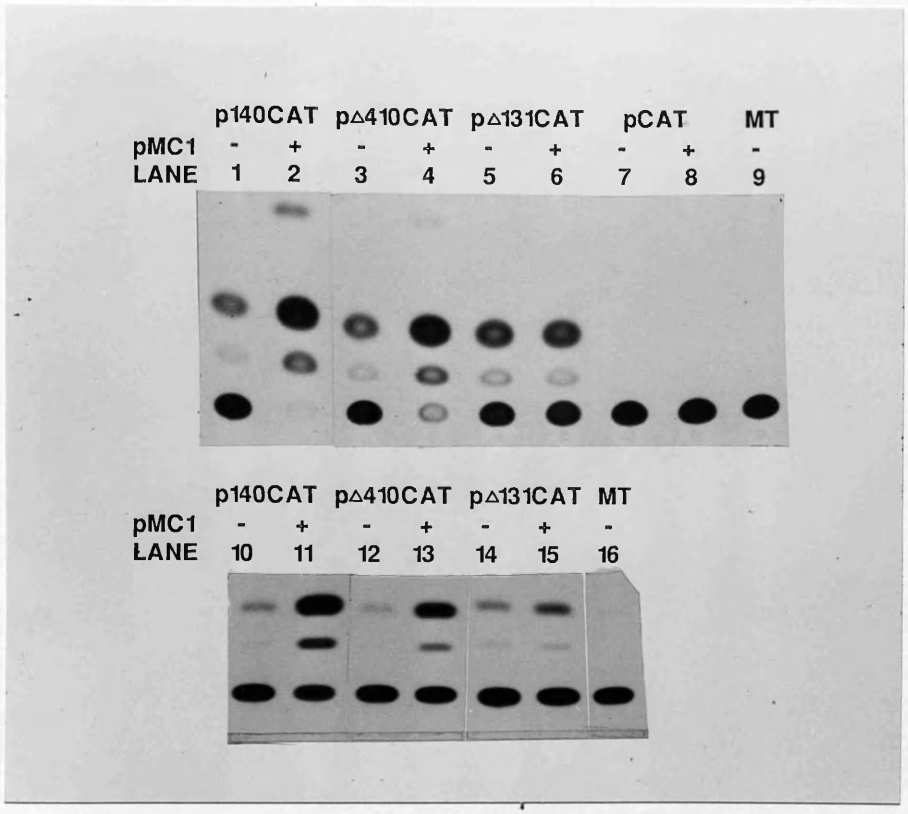

p140CAT pΔ410CAT pΔ131CAT pCAT MT
pMC1 - + - + - + - + -
LANE 1 2 3 4 5 6 7 8 9
BHK cells
p140CAT pΔ410CAT pΔ131CAT MT
pMC1 - + - + - + -
LANE 10 11 12 13 14 15 16
Hela cells

Table 4.1.A. BHK Cells

Normalised Percent. Acetlyation*

Plasmids	pMC1	Experiments							Ave
		1	2	3	4	5	6	7	
p140cat	-	28.5	2	26	19.5	23.5	8.5	5	
	+	1320	33	660	1510	1540	64.5	56	
	Stim^n	46.3	16.5	24.5	77.4	65.5	7.6	11.2	35
p140Δ410cat	-	15	24	9.5					
	+	600	460	50.5					
	Stim^n	40	19.2	5.3					22
p140Δ131cat	-	32	34	14					
	+	36	36	20					
	Stim^n	1.1	1	1.4					1

* BHK cells were transfected as described. The plasmid pFJ3, which expresses the B-galactosidase gene, was transfected in all experiments as an internal control. The volumes of extracts used in CAT assays were then adjusted on the basis of the B-galactosidase results in order to reduce the effect of variability between individual transfections.

Table 4.1.B. Hela Cells

Plasmids	pMC1	Normalised Percent. Acetylation* Experiments				Ave.
p140cat	-	2	2	1	1	
	+	46	62	19.5	23	
	Stim^{n}	**23**	**31**	**19.5**	**23**	**24.1**
p140Δ410cat	-	1	1			
	+	18	12			
	Stim^{n}	**18**	**12**			**15**
p140Δ131cat	-	2	2			
	+	4	5			
	Stim^{n}	**2**	**2.5**			**2.3**

* HeLa cells were transfected as described, again the plasmid pFJ3 was used as an internal control and volumes of extract of the test plasmids were adjusted on the basis of the B-galactosidase activity measured.

comparison between the regulatory regions of orf 62, those of its HSV homologue, IE gene 3, and those of its functional homologue in HCMV, the major IE gene. It was not intended to extrapolate any biological significance from this result, especially given the fact that autoregulation restricts the expression of all these genes during the lytic cycle. However, given the problems encountered detecting baseline expression from the VZV promoter, it was felt that a more formal comparison amongst these regulatory sequences would be informative. Thus, a series of short term transfection assays were undertaken to compare pl40cat with pIE3CAT and pRR55 in which the HSV IE3 and the HCMV IE control sequences, respectively, direct the expression of CAT. The results of this experiment (figure 4.9.) show that the VZV sequences were much less active in directing CAT expression; it was necessary to dilute extracts of cells transfected with pIE3CAT and pRR55 250-fold to obtain comparable enzyme levels. Thus in this direct comparison, expression of CAT from the VZV major IE gene was more than two orders of magnitude lower than its HSV and HCMV counterparts even in the presence of Vmw65, and in its absence the figure increased to over three orders of magnitude.

4.2.4. The Effect of Leader Sequences on Transcription.

The next stage in the analysis of the VZV orf62 regulatory region was to determine the sequences responsible for the apparent difference in the activities of pl40cat and pTM15cat. As shown (figure 4.4.) pl40cat contains additional VZV sequences both 3',+55 to +20, and 5', -783 to -1143, to those present in pTM15cat. Evidence gained from the deletions of pl40cat strongly argued that the 5' sequences were not responsible for the differences in expression, as detectable levels of CAT activity were produced by both pl40Δ410cat and pl40Δ131cat, (Figure 4.8.). Therefore residues from +55 to +20 region were implicated. To clarify this situation two further plasmids were constructed in which fragments encoding the proximal regulatory sequences of pl40cat were transferred to pTM15cat, and vice versa. This was achieved using an AflIII site at -35 and the EcoRI site 200bp within the CAT gene of

Figure 4.9. Comparison of IE Control Sequences of HSV-1, HCMV and VZV

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 is a mock transfected track. In lanes marked + (2, 5 and 7), reporter plasmids were co-transfected with pMC1 while in lanes marked - (1, 4 and 6) pUC18 was added in order to equalise plasmid quantities.


p140CAT
CON
pRR55
pIE3CAT
pMC1 - + - - + - +
DIL^n x 1 1 1 250 250 250 250
LANE 1 2 3 4 5 6 7

each plasmid. The new plasmids produced by this exchange were named pl40Δ781cat and pTM15+cat, (Figure 4.4.). Experiments comparing all four plasmids in BHK cells are shown in table 4.2.and figure 4.10. These results show that plasmids pTM15cat and pl40Δ781cat behaved in an equivalent manner, as did pl40cat and pTM15+cat, suggesting that sequences 3' to +20 had no effect on basal activity. Stimulation of transcription by pMC1 resulted in greater discrepancies within each set of comparable plasmids but no significant trends emerged. One notable feature of these experiments was that transfections with plasmids pl40Δ781cat and pTM15cat yielded considerably less activity in the absence of pMC1 than those which encoded the sequences between -781 and -1146. This finding did not correlate with results of experiments comparing plasmids pl40Δ410cat and pl40Δ131cat with pl40cat in which CAT activity with and without pMC1 was similar. The reasons for this apparent discrepancy are not clear.

The plasmids pTM15+cat and pl40Δ781cat were constructed to test the proposition that the differences between the activities of pl40cat and pTM15 cat resulted from the presence of sequences between +55 and +20. This was shown not to be the case. The absolute activities of the plasmids pl40Δ781cat and pTM15cat, which lack sequences between -781 and -1143, were considerably less than those of pl40cat and pTM15+cat which contain these sequences. The reasons for this differences are not clear as pl40Δ410cat, which lacks a further 374 bases, produced values closer to those of pl40cat (compare tables 4.1. and 4.2. experiments 1 and 2).

4.2.5. The Effect of Adenovirus E1A products on the orf62 Promoter.

A final set of transfections was performed to assess the effect of the adenovirus E1A transactivator protein on the control sequences of orf62. The reason for carrying out this experiment was the presence of the sequence motif ACAGCAGT at -71 to -79. This sequence is a 7 out of 8 match for the CRE or ATF binding site, ACTGCAGT (section 1.5.2.1.). Binding by members of this protein family has been shown to mediate responsiveness to the E1A gene

Figure 4.10. Effect of 5'Untranslated Sequences on Transcriptional Efficiency.

Plasmids pl40Δ781cat (lanes 1 and 2), pl40cat (lanes 3 and 4), pTM15cat (lanes 5 and 6) and pTM15+cat (lanes 7 and 8) were transfected into BHK cells in the pres ence (lanes 2, 4, 6 and 8) or absence (lanes 1, 3, 5, 7) of pMC1. Total quantities of plasmids were kept constant using pUC18. B-galactosidase assays were used to normalise CAT assays for transfection efficiency. Quantitations of CAT activity from this and other equivalent experiments are shown in Table 4.2.

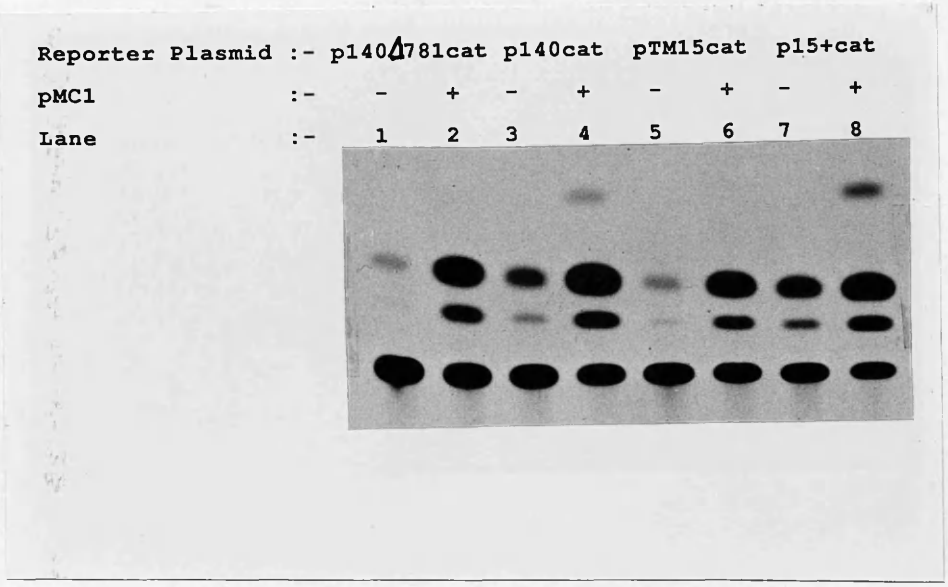

Reporter Plasmid :- p140Δ781cat p140cat pTM15cat p15+cat
pMC1 :- - + - + - + - +
Lane :- 1 2 3 4 5 6 7 8

Table 4.2.

Plasmids	pMC1	Normalised Percent. Acetylation* Experiments 1	2	3	4	Ave.
p140cat	-	28.5	-	8.5	5	
	+	1320	-	64.5	56	
	Stim^n	**46.3**	**-**	**7.8**	**11.2**	**21.8**
p140Δ781 cat	-	4	3	1	0.8	
	+	270	200	26	17.5	
	Stim^n	**67.5**	**66.7**	**26**	**21.9**	**45.5**
pTM15+cat	-	38	61	4	3	
	+	660	880	51.5	47	
	Stim^n	**17.4**	**14.4**	**12.9**	**15.7**	**15.1**
pTM15cat	-	2	2	1	-	
	+	19	34	27	-	
	Stim^n	**9.5**	**17**	**27**	**-**	**17.8**

* Reporter plasmids shown were transfected into BHK cells with equal quantities of the control plasmid pFJ3 which encodes the B-galactosidase gene. The quantity of extract used for determination of CAT activity was adjusted to equalise these values thus controlling for transfection efficiency. The values are expressed as counts per minute x 10^{-3}

product. Thus, the responsiveness of the orf62 promoter to E1A gene products was intended to provide functional corroboration for the sequence and protein binding data (figure 4.14) that suggested that members of the ATF/CRE protein family were involved in the control of the expression of orf62. The transactivation function of E1A has been mapped to a 46 amino acid region between residues 243 and 289 (section 1.5.2.4.). Of the two main mRNAs encoded by the E1A gene, only the 13S component contains this transactivation domain, whereas the smaller 12S component does not. Plasmids that express the "12S" or "13S" gene products were obtained from Dr. N. Jones. These plasmids were cotransfected with p140cat and deleted derivatives. The results of this experiment (figure 4.11.) show that co-transfection of a plasmid expressing the 13S component with p140CAT results in a 10-fold increase in CAT activity. This effect also occured when p140Δ410CAT and p140Δ131CAT were tested, both plasmids being stimulated to a similar extent. Thus sequences between +55 and -131 mediate responsiveness of the orf62 control sequences to the "13S" E1A gene product, but the exact target for stimulation (thought to be the ACAGCAGT or the TATA element) has not been identified.

4.3. Protein Binding Studies

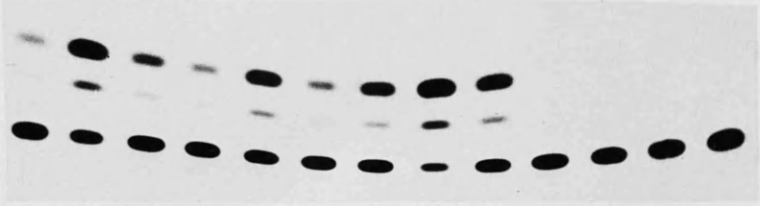
4.3.1. General Analysis

To complement and extend the functional assays, attempts were made to locate and characterise protein binding sequences in the orf62 regulatory region. Gel retardation assays were used for initial investigations. Fragments derived from restriction enzyme digests designed to cover the entire regulatory region were radiolabelled and incubated with nuclear extracts from HeLa cells. The results of a series of such assays are shown in figure 4.12. Two main regions of protein binding activity were detected. The first, lane 6, is located between the SalI and the XhoI sites at -409 and -130 respectively, and was named Region B. It corresponds to the region shown by the functional

Figure 4.11. Effects of the Products of the E1A Gene on orf62 Control Sequences.

Plasmids p140cat, p140 410cat, p140 131cat and pBLW2 were co-transfected with plasmids expressing the 289 amino acid product of the adenovirus 5 E1A gene (lanes 2, 5, 8 and 11), 243 amino acids product of the same gene (lanes 1, 4, 7 and 10) and pUC18 lanes (lanes 3, 6, 9 and 12). On this occasion no reporter plasmid was used to normalise transfection efficiency but the amount of protein added to individual reactions was controlled by Bradford assay. The relative CAT activity is given below the assays.

Reporter Plasmid	:-	p140CAT			p140 410CAT			p140 131CAT			pBLW2			MT
Cotransfected with	:-	12S	13S	pUC	12S	13S	pUC	12S	13S	pUC	12S	13S	pUC	-
Lane	:-	1	2	3	4	5	6	7	8	9	10	11	12	13

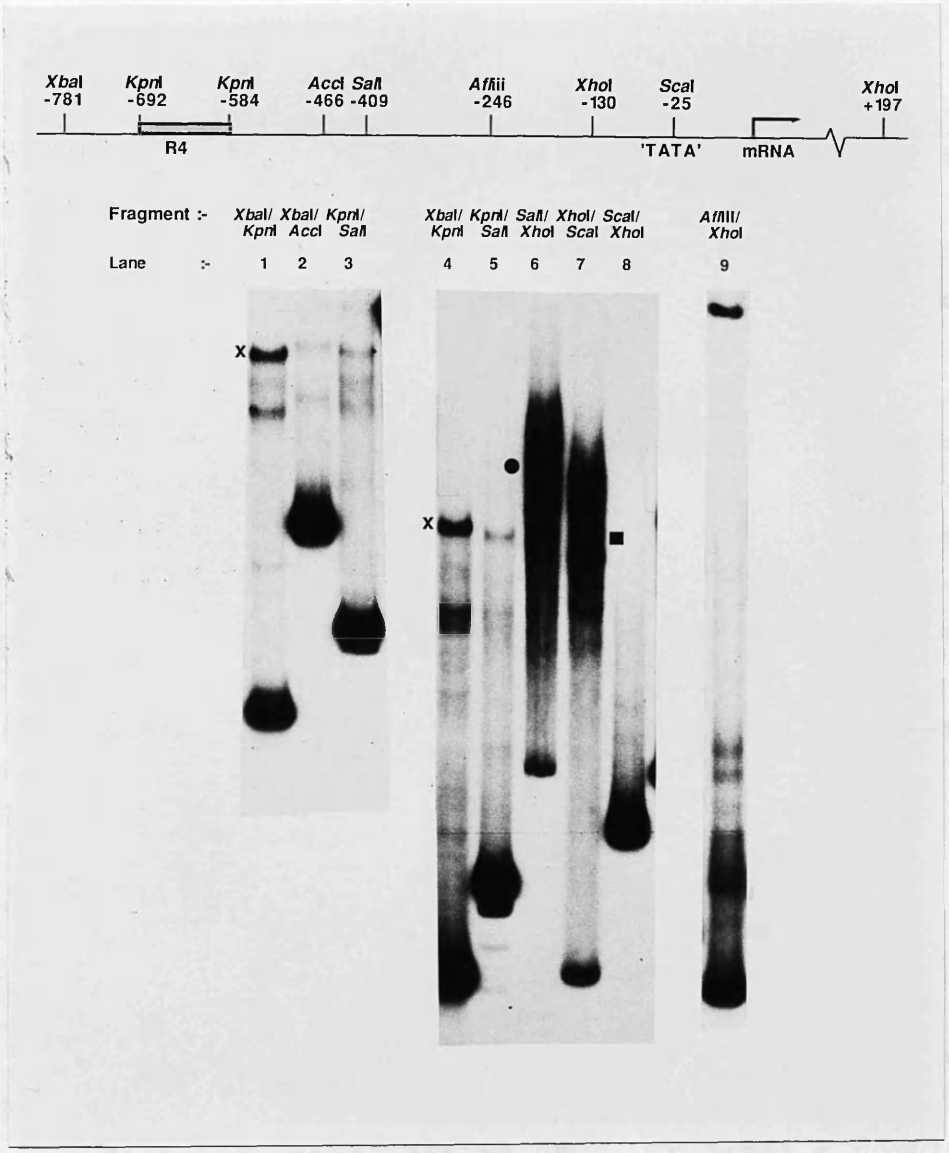


CAT Activity Normalised	:-	8	200	23.5	15	190	13.5	37	340	49	0.3	0.9	0.5	0.1

Abig 9 doc.

Figure 4.12. Gel Retardation Assays Using DNA Fragments Within the Putative Control Regions of orf62

The fragments assayed were derived from plasmid pVZVDH which was digested with the restriction endonucleases shown. The positions of the relevant cleavage sites are shown diagrammatically above the assays. Complexes that were subjected to further analysis were detected in lanes 6 and 7 marked ● and ■ respectively. The XbaI/KpnI fragment (lanes 1 and 4) consistently generated a retarded complex marked X.


XbaI -781
KpnI -692
KpnI -584
AccI -466
SalI -409
AffiII -246
XhoI -130
ScaI -25
XhoI +197
R4
'TATA'
mRNA
Fragment :-
XbaI/ KpnI
XbaI/ AccI
KpnI/ SalI
XbaI/ KpnI
KpnI/ SalI
SalI/ XhoI
XhoI/ ScaI
ScaI/ XhoI
AffIII/ XhoI
Lane :-
1 2 3 4 5 6 7 8 9
X
X

analysis to respond to stimulation by Vmw65. A further fragment cleaved with AflIII and XhoI from this region, lane 9 representing sequences -246 to -130, failed to direct complex formation, suggesting that the binding site(s) responsible for the retarded bands lies between -246 and -409, a result confirmed by subsequent experiments, (section 4.3.3.). The second fragment that results in prominent complex formation consists of sequences between -130 and -25 produced by XhoI and ScaI. This corresponds to the baseline promoter region of the functional studies and was named Region A. The complex formed at Region A can be resolved into two main parts, the upper being a diffuse area of radiodensity and the lower, a more discrete band.

Other fragments, notably those containing sequences between -584 and -784, appeared to interact with proteins resulting in retarded bands (lanes 1 and 4), however, these bands were of much lower intensity than those of lanes 6 and 7, and in view of the results of the functional studies, were not investigated further during the course of the work presented here. Nevertheless, the results suggest that specific protein binding occurs at this interesting region of the VZV genome.

4.3.2. Region A - The Proposed Promoter Fragment

In order to define the locations of the binding activities of Region A further, subfragments from this region were radiolabelled and assayed as shown in figure 4.13. The fragment assayed in lane 1 was cleaved with AflIII and SspI and spans -246 to -90. It binds a complex with a similar appearance and mobility, but apparently, in this experiment, lower affinity, than the lower complex formed by the fragment encoding the whole of region A (lane 2). Since, sequences between -246 and -130 had already been shown not to form retarded complexes (Figure 4.12., lane 9), this localises a potential binding site to between the XhoI site at -131 and the SspI site at -91. Lane 3 shows complexes formed by a SspI to ScaI fragment spanning -90 to -35 and representing the 3' portion of Region A. A complicated, or poorly resolved array of bands are formed on this fragment, which do not resemble complexes formed by the

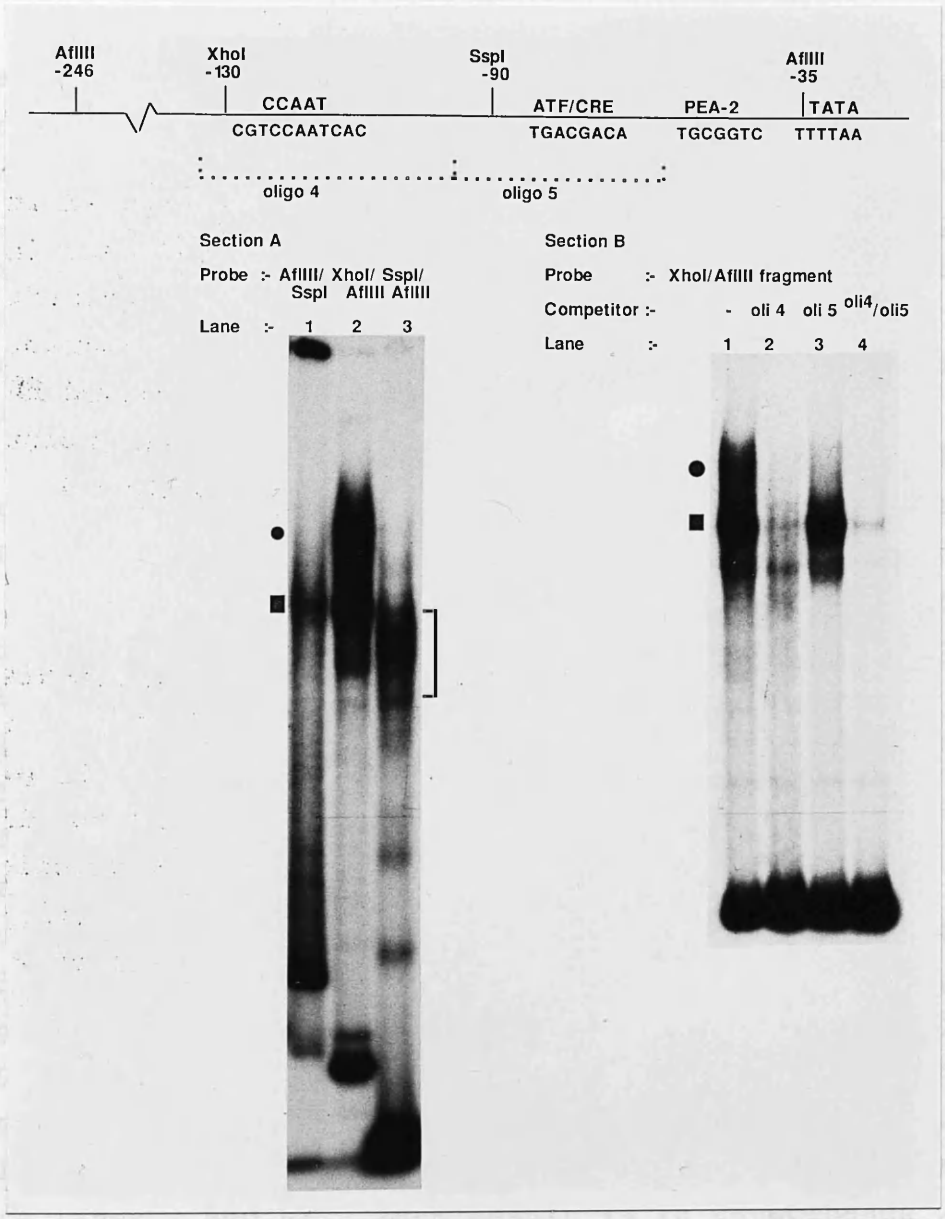
Figure 4.13. Analysis of Promoter Fragment: I.

Section A.

The promoter fragment (-131 to -30) was analysed in two parts. Residues -131 to -87 were analysed within a larger fragment (Lane 1), while residues -87 to -35 were assayed in isolation (Lane 3). The pattern of binding to the entire fragment is reproduced for comparison (Lane 2). Complexes marked ■ were common to Lanes 1 and 2, the complex marked ● was unique to lane 2 and the complex marked] was unique to lane 3.

Section B.

Double stranded oligonucleotides 4 and 5 (Appendix 3) were incubated alone (Lanes 2 and 3 respectively) or in combination (Lane 4), with HeLa cell nuclear extract, prior to the addition of [^{32}]P-labelled fragment (residues -131 to -35). In the absence of competitor two complexes could be discerned, one, marked ● , migrated slowly, the other, marked ■ , migrated more quickly. Oligonucleotides were added at approximately 100 fold molar excess.


AflIII
-246
XhoI
-130
SspI
-90
AflIII
-35
CCAAT
CGTCCAATCAC
ATF/CRE
TGACGACA
PEA-2
TGCGGTC
TATA
TTTTAA
oligo 4
oligo 5
Section A
Probe :- AflIII/ SspI
XhoI/ AflIII
SspI/ AflIII
Lane :- 1 2 3
Section B
Probe :- XhoI/AflIII fragment
Competitor :- - oli 4 oli 5 oli4/oli5
Lane :- 1 2 3 4

complete Region A fragment. Thus, neither part of Region A alone could reproduce the binding activity of the entire fragment.

Two possible explanations can be proposed to explain these findings. The binding activity seen in lane 2 could, at least in part, be dependent on the integrity of a site situated around -91, and cleavage at or close to this site could disrupt this motif and thus prevent binding. Alternatively the pattern could result from additive effects of proteins binding at two unrelated sites, on the large fragment. Thus the lower complex would result from the binding of a single protein to the fragment while the upper complex would result from the occupation of both sites by proteins. Separate fragments would not then reproduce this pattern. These possibilities are obviously not mutually exclusive.

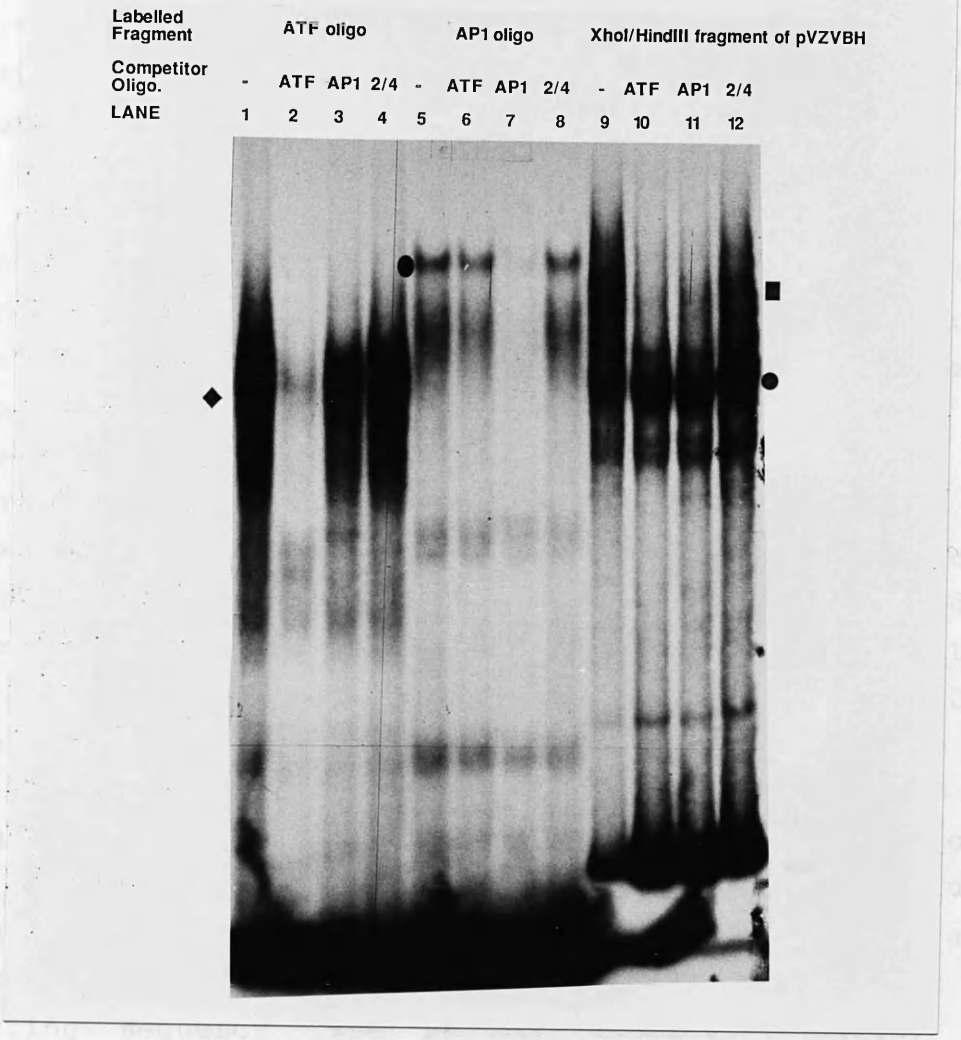
A search of this Region A for known cis-acting motifs revealed three possible protein binding sites centered at -53 (PEAII), -78 (ATF/CRE) and -115 (CCAAT). The fact that PEAII is an enhancer binding protein that to date has only been detected in mouse cell extracts led to the suspicion that the latter two factors were more likely to be of significance. Oligonucleotides encoding these sites and their flanking sequences were designed and synthesised. The use of these oligonucleotides as competitors in gel retardation assays is shown in figure 4.13 section B. Preincubation with oligo 5, which includes the ATF site (lane 3), resulted in the disappearance of the upper complex leaving the lower one unaffected. Oligo 4, which includes the CCAAT box homology competes for the lower band, (lane 2), however, this oligonucleotide also appears to reduce overall binding leaving only a small amount of diffuse bands (compare lanes 2 and 4). This result is in concordance with that shown in figure 4.13. section A in suggesting that the lower band in lane 2 is largely the result of protein binding to the distal half of region A. Competition with both oligonucleotides (section B lane 4) left little binding activity. Taken together, the results of figure 4.13. sections A and B suggested the presence of two protein binding sites in Region A. The most likely candidates for these are the CCAAT box motif present at -115 and the

CRE/ATF binding site at -78. However, a simple model, in which two distinct binding sites of equal affinity each interact independently with a protein, does not entirely explain the findings as competition with oligo 4 left little binding activity, apparently less than that present in the upper complex alone. This suggests the possibility of an interaction between the two proteins whereby binding to the region encoded by oligo 4 facilitates binding to the site in the proximal part of the fragment.

Attempts were then made to identify the binding proteins. Oligonucleotides known to encode binding sites for ATF and APl, along with an oligonucleotide which contained two mutations that inactivate the ATF binding site (2/4), were kindly provided by Dr. N. Jones. The result of an experiment, in which these oligonucleotides were used both as probes and competitors in gel retardation assays is shown in figure 4.14. First, use of the ATF oligonucleotide as a probe revealed a single, relatively diffuse, complex (lane 1). Use of the same oligonucleotide as a competitor removed this complex almost completely (lane 2), the APl oligonucleotide competed partially for the complex (lane 3), and oligo 2/4, in which the ATF binding site had been mutated, (Appendix 3) failed to compete for the ATF complex (lane 4). The use of the APl oligonucleotide as a probe (lanes 5-8) resulted in a much lower level of protein binding. However, the complex which was produced was removed completely by an excess of the APl oligonucleotide (lane 7), the ATF oligonucleotide competed to a small extent (lane 6) and oligo 2/4 again failed to compete (lane 8). These data are consistent with those already reported using the same oligonucleotides (Hurst and Jones, 1987). Finally, using Region A as a probe the dual complex was again produced in the absence of competitors (lane 9). Addition of an excess of the ATF oligonucleotide effectively competed for the upper complex (lane 10). The APl oligonucleotide also competed, though slightly less efficiently (lane 11). Again the oligo 2/4 failed to compete to any measurable extent (lane 12). These data suggest strongly that ATF, or members of that protein family, represents one of the binding activities associated with region A, since the upper complex formed by the fragment containing this region shows

Figure 4.14. Analysis of Promoter Fragment: II.

Protein binding to the promoter fragment (XhoI/HindIII of pVZVBH, residues -131 to -25) was compared with binding to oligonucleotides containing the ATF and AP-1 binding sites. These oligonucleotides, along with one (2/4) containing mutations known to prevent binding to the ATF site, were also used as competitors. The oligonucleotide containing the ATF site was used as a probe in Lanes 1 to 4, that containing the AP-1 site was used as a probe in lanes 5 to 8 and the -131 to -25 fragment of pVZVBH was used as a probe in lanes 9 to 12. Lanes 1, 5, and 9 contained no competitor oligonucleotides, the ATF oligonucleotide was added, prior to labelled fragment, in lanes 2, 6 and 10, the AP-1 oligonucleotide was added to lanes 3, 7,and 11 and the oligonucleotide containing the mutated ATF site, 2/4, was added to lanes 4, 8 and 12. All oligonucleotides were added at approximately 100 fold molar excess. The slower and faster migrating complexes were again marked ● and ■ respectively. The complexes present in the ATF and AP-1 tracks were marked ◆ and ▮ respectively.

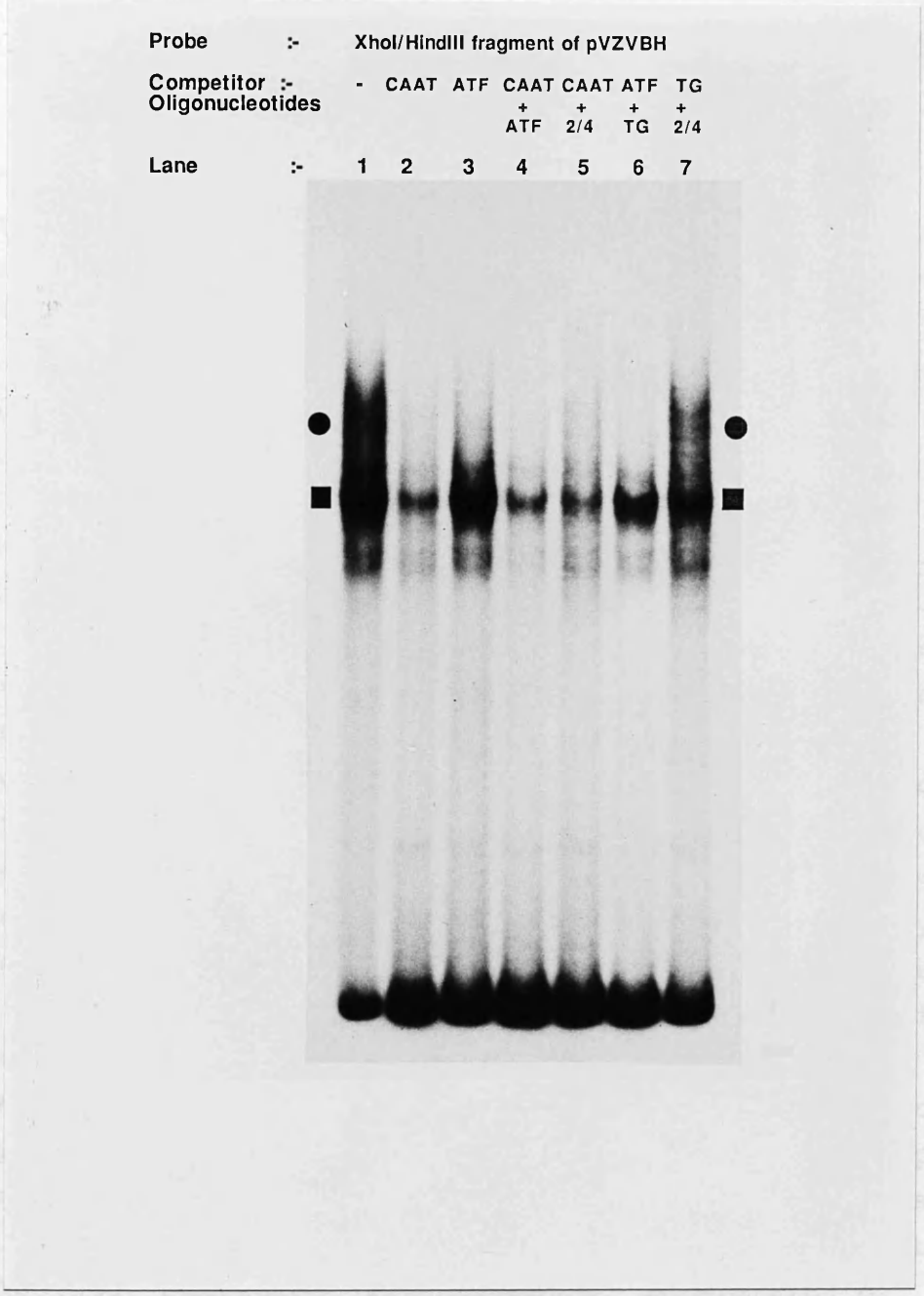

Labelled Fragment
ATF oligo
AP1 oligo
XhoI/HindIII fragment of pVZVBH
Competitor Oligo.
- ATF AP1 2/4 - ATF AP1 2/4 - ATF AP1 2/4
LANE
1 2 3 4 5 6 7 8 9 10 11 12

the same response to competition as the radiolabelled ATF oligonucleotide. It was also noted that competitors that remove the upper complex increased the intensity of the lower complex (compare lane 9 with lanes 10 and 11), again suggesting that the upper complex results from binding of the fragment by two or more proteins. The removal of one protein thus results in more fragment bound by a single protein and thus increased intensity of the faster migrating complex. Also the double complex runs more slowly than the ATF complex.

Further evidence towards identifying the binding species was produced by another competition experiment (figure 4.15). Again Region A was used as the probe. Four oligonucleotides, in two pairs, were used as competitors. Two of these contained the motifs present in Region A represented by the HSV TK CCAAT box and the ATF oligonucleotide used previously. The others were the mutated ATF oligonucleotide (2/4) used previously and an oligonucleotide which encoded the TAATGARAT motif found upstream of HSV IE genes. Lane 1 shows complex without competitor oligonucleotides. Lane 2, in which the HSV CCAAT box alone was the competitor, yielded a pattern similar to that seen in figure 4.13., section B lane 2, when oligo 4 was the competitor, although the TK CCAAT box appeared to be less effective in competing for the complex. This failure of full competition is not surprising given the number and diversity of proteins which are known to bind to this cis-acting sequence (see section 1.5.2.3.). Again, competition of protein binding from this site left little evidence of ATF binding, though comparison of lanes 4, 5 and 6 does appear to show a diffuse complex in the lane which lacks the ATF competitor (lane 5). This result suggests that the absence of the CCAAT box binding activity in some way reduces the affinity of the ATF binding species for its site. The converse is not true, as shown in lanes 3 and 6, since the presence of the oligonucleotide encoding the ATF binding site again results in the removal of the upper complex and an increase in the intensity of the lower, CCAAT box related, complex. Finally, competition with both specific oligonucleotides (lane 6) resulted in the removal

Figure 4.15. Analysis of the Promoter Fragment: III.

The XhoI/HindIII fragment of pVZVBH (residues -131 to -20) again served as the probe and was incubated with HeLa cell nuclear extract. A 100-fold molar excess of oligonucleotides containing the CCAAT box motif (Lanes 2, 4 and 5), the ATF/CRE motif (Lanes 3, 4, and 6), the mutated ATF/CRE motif (Lanes 5 and 7) and the TAATGARAT motif (Lanes 6 and 7). As before the faster and slower migrating complexes are marked ■ and ● , respectively. Sequences of the competitor oligonucleotides are given in Appendix 3.


Probe :- XhoI/HindIII fragment of pVZVBH
Competitor Oligonucleotides :- - CAAT ATF CAAT + ATF CAAT + 2/4 ATF + TG TG + 2/4
Lane :- 1 2 3 4 5 6 7

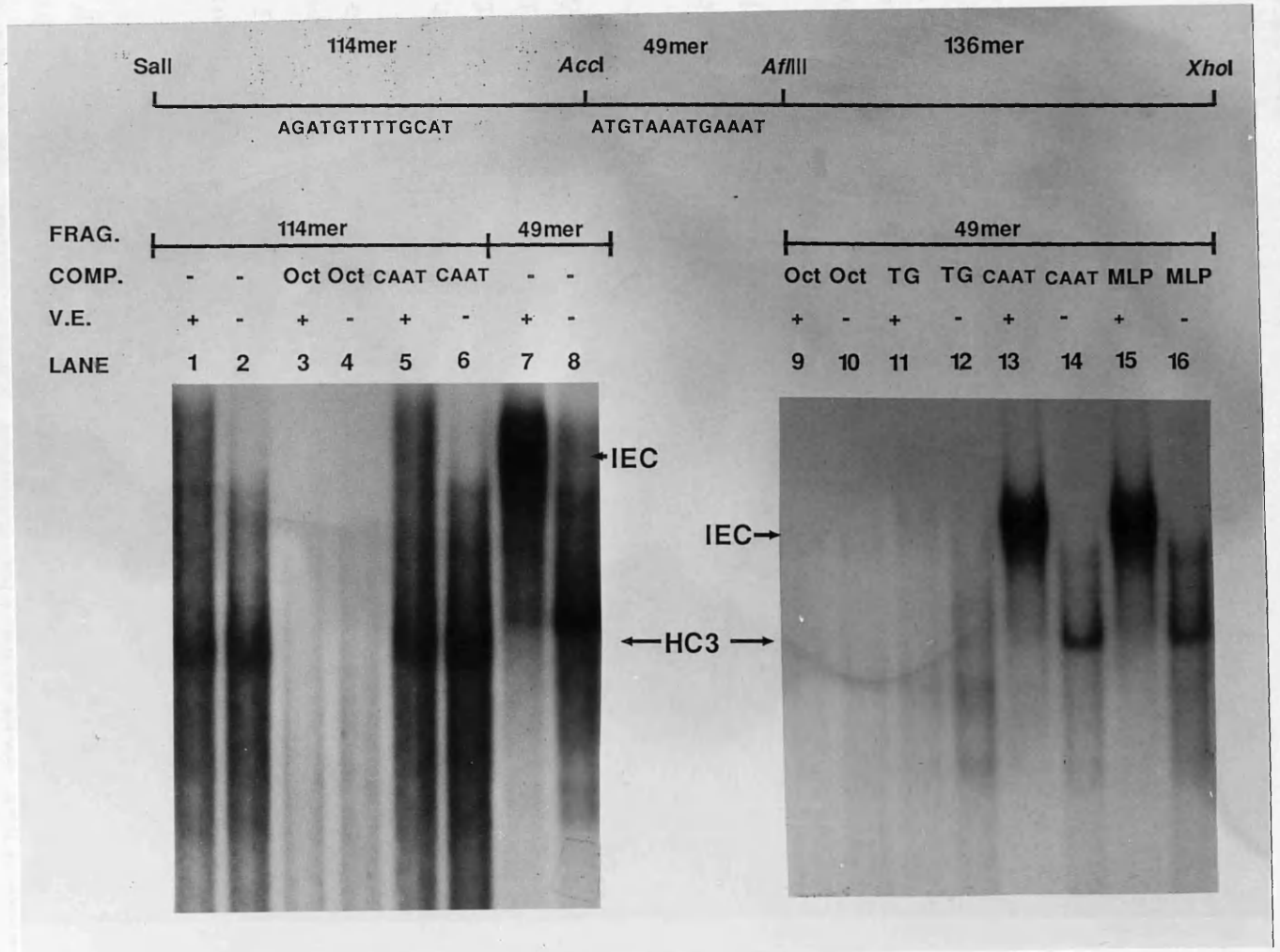
of most of the binding activity, while the non-specific oligonucleotides had little effect (lane 7).

4.3.3. 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 recognise the octamer element ATGCAAAT as well as TAATGARAT (section 1.4.2.). Analysis of the region upstream of the TATA box reveals two sites which match the octamer element closely (figure 4.1). Site I (TTTTGCAT) is centered at -345 and matches the octamer element at 7 out of 8 positions in the opposite orientation. Site II (ATGTAAATGAAAT) is centered at -256, and is a more complicated motif representing a combination of the octamer sequence (7 out of 8 matches) and the TAATGARAT motif (8 out of 9 matches). Both of these sites are within nucleotides -409 to -130, 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 (figure 4.16.). Three radiolabelled fragments from the region -410 to -131 in the VZV IE promoter were tested in the assay. A 114 bp SalI/AccI fragment, containing TTTTGCAT, gave a complex (lane 2) equivalent to that denoted HC3 by Preston et al., (1988), and upon addition of herpes simplex virion extract, a very diffuse complex that migrated slowly into the gel was formed (lane 1). Production of both radiolabelled complexes was competed by addition of oligonucleotides containing ATGCAAAT (lanes 3 and 4), but not by an oligonucleotide containing the HSV-1 TK gene CCAAT motif (lanes 5 and 6). A 49 bp AccI/AflIII 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 9-12), but oligonucleotides containing CCAAT or adenovirus major late promoter (MLP) recognition sites did not (lanes 13-16). An AflIII/XhoI fragment comprising the remainder of

Figure 4.16. Gel Retardation Analysis of the Region Between -409 and -130.

Fragments containing the octamer motif (114 bp) and the octamer/TAATGARAT motif (49 bp) were incubated with HeLa cell nuclear extract. HSV-1 virion extract 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.


SalI
114mer
AccI
49mer
AflIII
136mer
XhoI
AGATGTTTTGCAT
ATGTAAATGAAAT
FRAG.
114mer
49mer
49mer
COMP.
- - Oct Oct CAAT CAAT - -
Oct Oct TG TG CAAT CAAT MLP MLP
V.E.
+ - + - + - + -
+ - + - + - + -
LANE
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
IEC
HC3
IEC

the -409 to -130 region failed to form any protein-DNA complexes (figure 4. 12. lane 9). Thus the two subfragments of the -409 to -130 region that bind proteins both interact with Vmw65, but IEC forms far more efficiently on the AccI/AflIII fragment that contains a 8/9 match to TAATGARAT. The activity of the very weak IEC formation by the 114bp fragment that contains the octamer motif alone, in terms of the stimulation of transcription, is questionable and thus lends weight to previous data which showed that octamer motifs alone are ineffective in Vmw65 mediated transcriptional stimulation (section 1.4.3.).

The gel retardation assays (figure 4. 16) provide strong evidence that the SalI-AccI and the AccI-AflIII fragments bind the protein Oct-1, The AccI-AflIII fragment also forms a tertiary complex. In order to confirm the location of the binding activity detected by the gel retardation analyses, DNAase I protection analysis of the AccI-XhoI fragment was undertaken (4. 17.). Protection from DNAase I digestion was present at the ATGTAAATGAAAT motif both in the presence and absence of partially purified Vmw65. No other differences from the control lane containing fragment alone were detected. Comparisons of the protection pattern between lanes 2 and 3 (with and without partially purified Vmw65) shows a small extension of the footprint towards the "GARAT" portion of the motif.

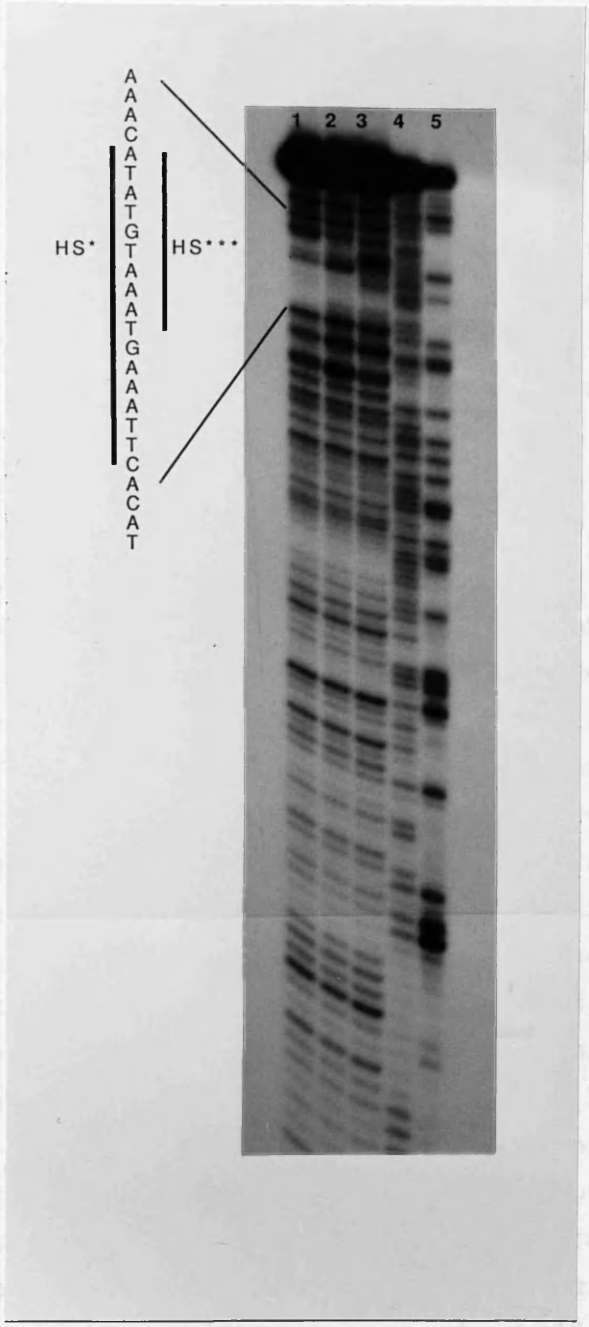
By analogy with the well characterised HSV-1 system, it is clear that the two elements with homology to the octamer and/or TAATGARAT are responsible for the observed stimulation of expression from the VZV major IE promoter by Vmw65, and it is likely that the element ATGTAAATGAAAT is of major importance for the effect.

4.4. Activity of VZV orf10

It was of interest to determine whether the VZV homologue of Vmw65, orf10, could also stimulate transcription from the VZV major IE gene. To investigate this possibility, the coding sequences of Vmw65 in the plasmid pMC1 were truncated by removing sequences between amino acids 413 and 471 which encode the majority of the

Figure 4.17. DNAaseI Footprinting Analysis of a 165bp AccI/XhoI (-296 to -131) Fragment.

Tracks 4 and 5 are G and G+A sequencing tracks. Prior to DNAaseI treatment, fragment in track 1 was incubated with HeLa cell extract and virion extract, fragment in track 2 with HeLa cell extract, while track 3 represents free fragment alone. Dark bars represent the sequences protected and hypersensitive sites are labelled HS.


1 2 3 4 5
AAACATATGTAAATGAAATTCACAT
HS*
HS***

acidic region (figure 4.18.) to form the plasmid pMC1 15-17, and then replaced by those of orf10 to form the plasmid pMCORF10. Figure 4.19. shows a comparison of the ability of pMC1 and pMCORF10 to stimulate transcription from the plasmids p140cat, pTM14gal and pTM8, which encodes the B-galactosidase gene directed by HSV IE3 control sequences (Appendix 2). In both of these examples pMCORF10 failed to stimulate expression of the reporter gene products, whereas a 10-30 fold increase in activity was observed upon co-transfection of pMC1. The results show that pMCORF10 fails to stimulate transcription. Comparison of the sequences of orf10 and Vmw65 had shown considerable 135% similarity in their amino-terminal domains (figure 4.18.). However, Vmw65 encodes a further region of 78 amino acids at its carboxy-terminus whose major characteristic is its acidity, having a total charge of -19. Acidic regions had been shown to be important for the ability of a group of DNA binding proteins to stimulate transcription (section 1.5.4.). Thus the absence of the acidic region from the coding sequence of orf10 alone would be sufficient to explain its lack of transcriptional activity. However, in view of the existence of transcriptional activators which lack acidic activating (section 1.5.3.) regions and the possibility that roles other than transcriptional activation might be served by the formation of the IEC, it was still of considerable interest to determine whether the protein encoded by orf10 would form a complex analogous to IEC. The coding sequences of orf10 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 (Ace *et al.*, 1989) cleaved with BamHI was transcribed, to provide a transcript lacking the sequences encoding the acidic C-terminal tail but retaining the N-terminal 410 amino acids of Vmw65 that are homologous to orf10. 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

Figure 4.18. Optimal Alignment of the Amino Acid Sequences of HSV-1 Vmw65 and the VZV orf10 Product.

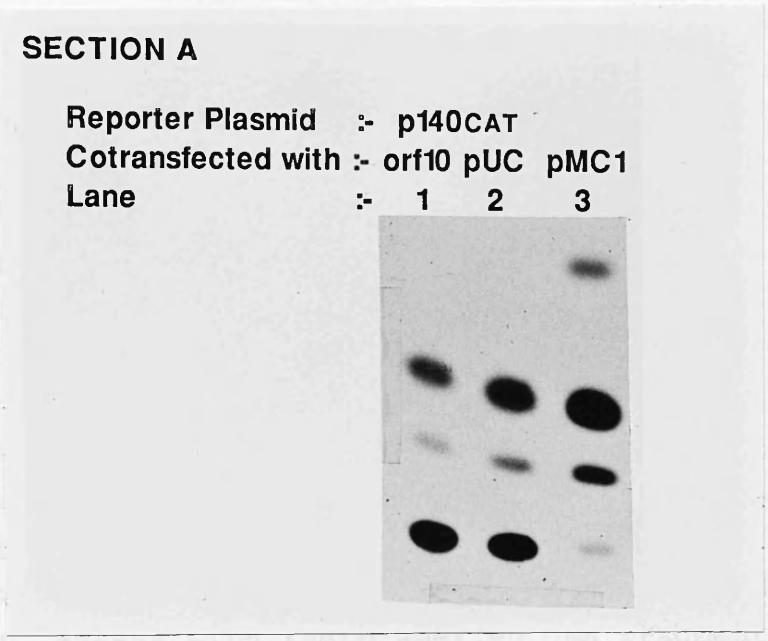
Amino acid sequences (adapted from Dalrymple et al., (1985)), are presented using the single letter code. The alignment was determined using the optimal alignment program of Taylor, (1984). Identical amino acids are denoted by *, acidic residues within the carboxy-terminal 78 amino acids are presented in bold script and the positions of the SalI site and the insertion mutants in14, in15 and in17 (Ace et al., 1988) relevant to this project are indicated.

```
HSV-1    1:-  MD..LLVDELFADMNADGASPPPPRPA..GGPKNTPAAPPLYATGRLSQA.QLMPSPPMPVPPA
              *   *        *              *          *       *   * *    ****    *
VZV      1:-  MECNLGTHEPSTDTWNRSKTEQAVVDAFDESLFGDVASDIGFETSLYSHAVKTAPSPPWVASPK


70   ALFNRLLDDLGFSAGPALCTMLDTWNEDLFSALPTNADLYRECKFLSTLPSDV...VEWGDAYVPERTQIDIRA
       *   *  ** ** ** *   * ********  * * ***     **  * **   *   *    *     *
75   ILYQQLIRDLDFSEGPRLLSCLETWNEDLFSCFPINEDLYSDMMVLSPDPDDVISTVSTKD..HVEMFNLTTR.


141  HGDVAFPTLPATRDGLGLYYEALSRFFHAELRAREESYRTVLANFCSALYRYLRASVRQLHRQAHNRGRDRDLG
       * *  *  *    **  *       *  ******* *   *   *    ***                 *
145  .GSVRLPSPPKQPTGLPAYVQEVQDSFTVELRAREEAYTKLLVTYCKSIIRYLQGTAKRTTIGLNIQNPDQKAY


215  EMLRATIADRYYRETARLARVLFLHLYLFLTREILWAAYAEQMMRPDLFDCLCCDLESWRQLAGLFQPFMFVNG
       **  *  ***** * *** * *****  ***  *  ** *   ** *  *       **    * *     *
219  TQLRQSILLRYYREVASLARLLYLHLYLTVTREFSWRLYASQSAHPDVFAALKFTWTERRQFRCAFHPVLCNHG


289  ALTVRGVPIEARRLRELNHIREHLNLPLVRSAATEEPGPALTTPPTLHGNQARASGYFMVLIRAKLDSYSSPTT
           * *  *  *** *  *  * *****    **   **   *       *  *     *  *** *
293  IVLLEGKPLTASA;REONYRRRELGLPLVRCGLVEENKSPLVQQPSFSVHLPRSVGFLTHHIKRKLDAYAVKHP


                                  in14                                SalI in15
363  SPSEAVMREHAYSRARTKNNYGSTIEGLLDLPDDDAPEEAGLAAPRLSFLPAGHTRRLSTAPPTDVSLGDELH
         *   * *       **** *             *   ** * *   *   *   *    ***   *
357  QEPRHVRADHPYAKVVENRNYGSSI.....EAMILAPPSPSEILPGDPPR....PPTCGFLTR


                                                 in17
437  LDGEDVAMAHADALDDFDLMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFRDALGIDEYGG
```

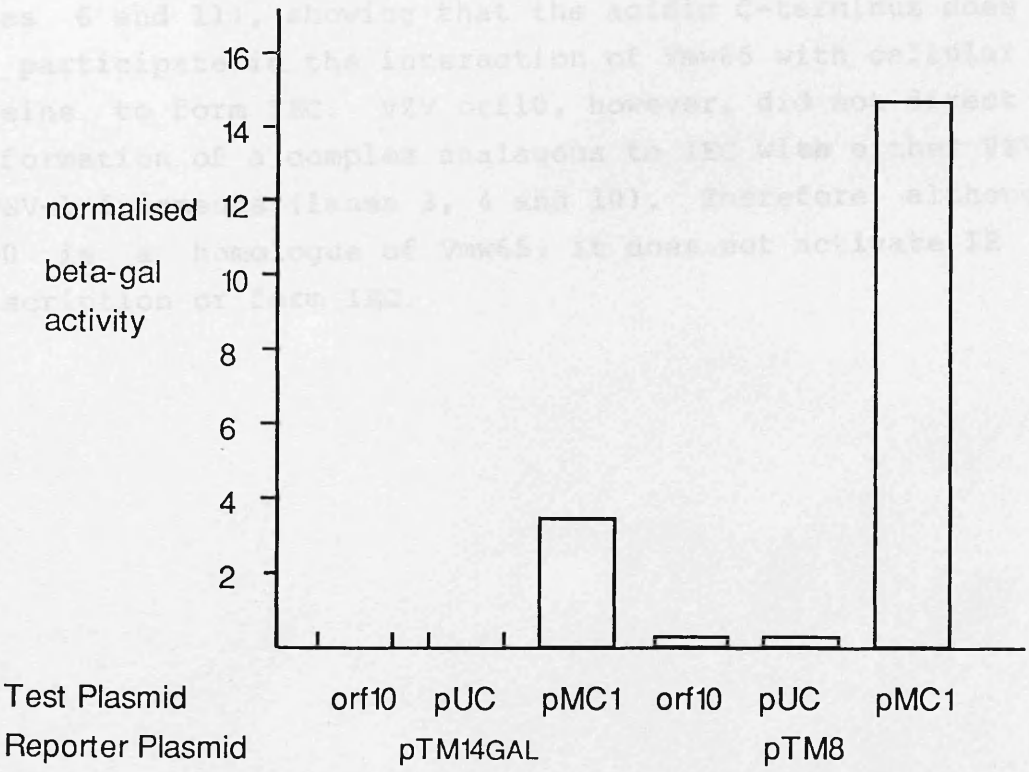
Figure 4.19. Transcriptional Activity of pMCORF10.

pMCORF10 was co-transfected with three reporter plasmids and its transcriptional activity was compared with that of equal quantities of pUC18 and pMC1. Section A shows the effects of pVZVORF10 (Lane 1), pUC18 (Lane 2) and pMC1 (Lane 3) on CAT gene expression from the plasmid p140cat. Section B shows the effect of the same three plasmids on B-galactosidase expression from the plasmids pTM14gal and pTM8.

SECTION A



SECTION B



translation products of the expected sizes upon SDS-PAGE (Figure 4.20.). Some discrepancy is noted between the predicted molecular weights of Vmw65 and the product of orf10 which are 54,000 and 43,000 respectively, and their apparent molecular weights of 63,000 and 54,000 respectively. This could be the result of post-translational modifications occuring in the rabbit reticulocyte lysate or some characteristic of the primary structure of the protein. In any case, it is not directly relevant to this experiment, the main purpose of which was simply to demonstrate that protein was produced. The differences in the intensities of the bands produced by the VZV and HSV orfs are largely due to the occurence of 3 times more methionine residues in HSV-1 Vmw65 than in VZV orf10. 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 (Figure 4.21). As expected, Vmw65 produced *in vitro* from pGEMTIF directed the formation of IEC when tested with the 49 bp AccI/AflIII 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. VZV orf10, however, did not direct the formation of a complex analagous to IEC with either VZV or HSV-1 fragments (lanes 3, 4 and 10). Therefore, although orf10 is a homologue of Vmw65, it does not activate IE transcription or form IEC.

Figure 4.20. In vitro Translation of Vmw65 and ORF10 Polypeptides.

RNA transcribed from pGEMTIFin15 after BamHI cleavage (lane 1), pGEMTIF (lane 2), pGEM2 (lane 3) and pGEMORF10A and B (lanes 4 and 5) all cleaved with HindIIIwere translated in vitro in the presence of [^{35}S]-methionine.

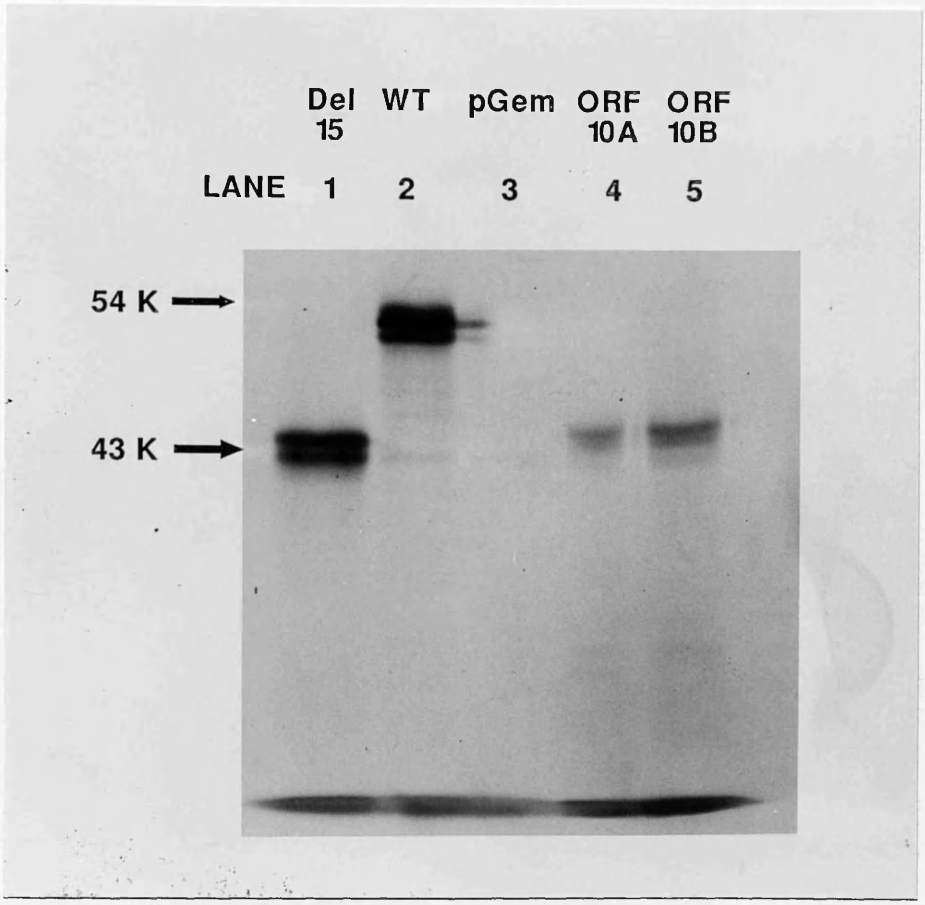
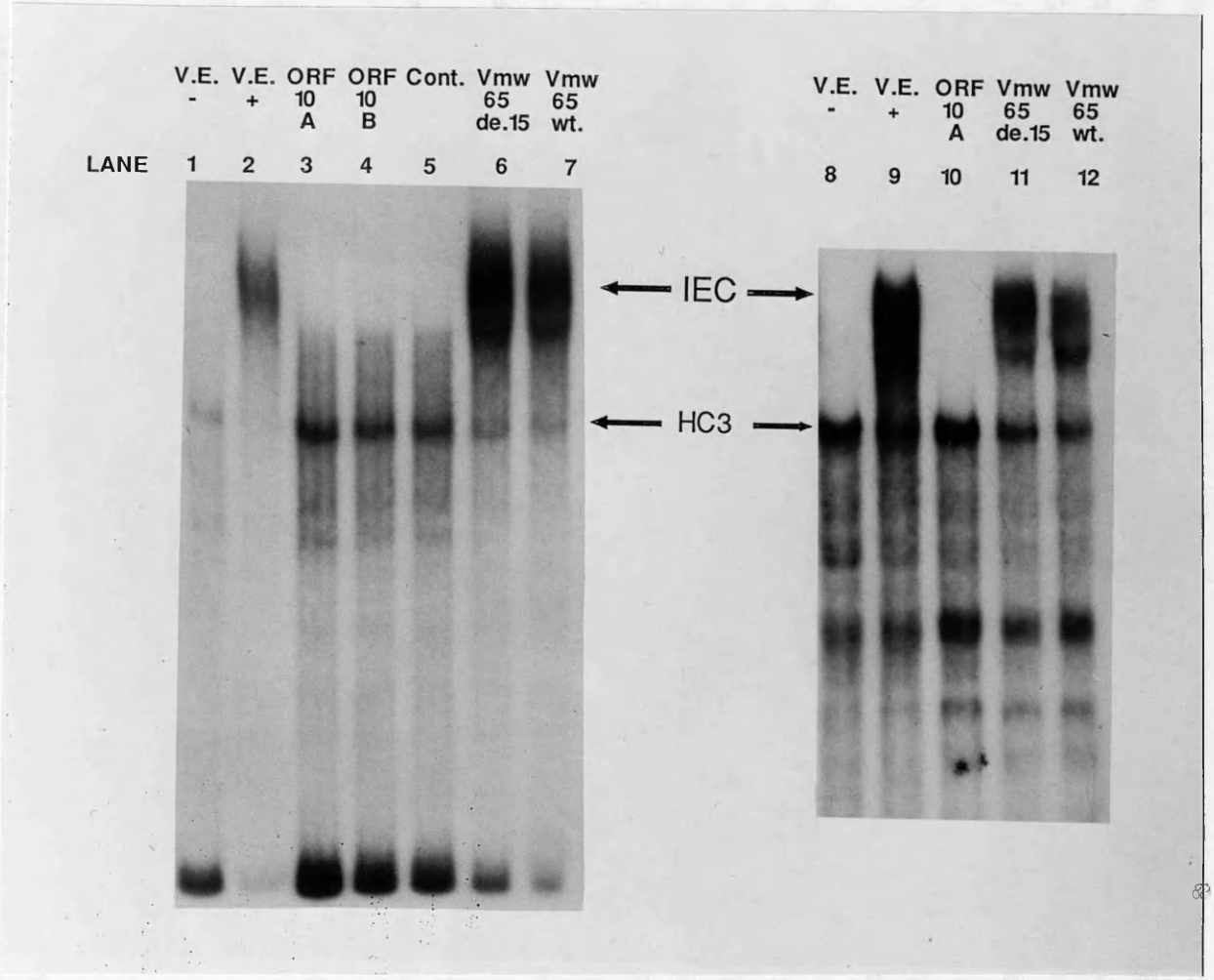

Del 15
WT
pGem
ORF 10A
ORF 10B
LANE 1 2 3 4 5
54 K
43 K

Figure 4.21. Gel Retardation Assay Using In vitro Synthesized Vmw65 and ORF10 Products.

Radiolabelled probes used in binding reactions were a 49bp AccI/AflIII fragment from the VZV major IE gene control sequences (lanes 1-7) and a 74 bp fragment from HSV-1 IE control sequences (lanes 8-12). 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 ORF10 (lanes 3, 4, and 10), pGEMTIF (lane 5), pGEMTIFin15 (lanes 6 and 11), or pGEMTIF (lanes 7 and 12). The positions of complexes IEC and HC3 are indicated.


V.E. -
V.E. +
ORF 10 A
ORF 10 B
Cont.
Vmw 65 de.15
Vmw 65 wt.
LANE 1 2 3 4 5 6 7
V.E. -
V.E. +
ORF 10 A
Vmw 65 de.15
Vmw 65 wt.
8 9 10 11 12
IEC
HC3

5. DISCUSSION

5.1. Cis-acting Sequences

For the purpose of discussion the sequence upstream of the proposed ATG of VZV orf62 can be divided into four sections. These are the 5' untranslated region, the promoter region (sequences -1 to -131), the Vmw65 responsive region (-131 to -410) and sequences present further upstream.

5.1.1. 5' Untranslated Region

Primer extension and S1 nuclease analyses revealed that the 5' untranslated region consists of 74 bases and lacks an intron. Further circumstantial evidence confirming this as the mRNA start site comes from the presence of a TATA box centred at -28 and functional regulatory elements further upstream.

Functional significance for sequences around the mRNA start site has been demonstrated in the HSV-1 system where a motif (ATCGTC), which spans the mRNA initiation site interacts with the IE-3 gene product Vmw175 (Beard et al., 1986; Faber and Wilcox, 1986; Kristie and Roizman, 1986a; Muller, 1987; Faber and Wilcox, 1988). This motif appears to be involved in the ability of Vmw175 to autoregulate its own expression (Gelman and Silverstein, 1987b; Muller, 1987; Roberts, 1988). Sequence similarity between the mRNA initiation sites of IE-3 and orf62 is not present and this feature correlates with the inability of 140K to down-regulate the expression of Vmw175 (Disney et al., manuscript in preparation). Recent, unpublished, results from G. Disney (manuscript in preparation) suggest that the product of orf62 does autoregulate its own expression but that sequences within the 5' untranslated region are not essential for this effect. Thus, orf62 can complement Vmw175 defective mutants (Section 1.3.2.2.), suggesting that the ability to transactivate is conserved both in terms of the mechanism of transactivation and the recognition of the essential genes whose expression depends on the presence of Vmw175. However the autoregulatory functions of the product

of orf62 and Vmw175 appear to be divergent, at least in terms of sequence recognition, if not also in their mechanism of action.

Mapping of the mRNA start site of orf62 also allows direct comparison with HSV-1 IE gene 3. Little conservation is apparent between the two sequences, the HSV-1 leader sequence being 301 bases compared to the 74 bases of the VZV leader. The only other VZV gene whose mRNA initiation site has been defined is that of the dPyK which was mapped by Davison and Scott (1986). It is located 410 bases upstream of the proposed initiation codon compared with a 5' untranslated region of 110 bases for the homologous HSV-1 gene (McKnight, 1980). Together, these data suggest that little conservation exists between the structures of the mRNA leader sequences of HSV-1 and VZV.

5.1.2. The Promoter

The promoter region, functionally defined by the plasmid p140Δ131cat, extends from the initiation site to -131. This fragment contains three recognised cis-acting elements, the TATA box (centred at -28), an ATF/CRE binding site (centred at -75) and a CCAAT box (centred at -116). In addition there is a sequence motif for the murine enhancer binding protein PEA-2 (TGCGGTC, centred at -52) though no binding activity related to this site was detected and the factor currently has not been detected in non-murine cells.

The proposed TATA box TTTTAA is present at -28. Comparison with the TATA sequences of the homologous genes in other α-herpes viruses; HSV-1 (ACTATAT), HSV-2 (ACTATAT), PRV (CTTATAA) and EHV-1 (GTATAAA - predicted), reveals little obvious conservation, amongst these motifs. The significance of differences in TATA box sequences is controversial. Recent evidence, gained using cloned yeast and purified human TFIID (the TATA binding factor, section 1.5.5.) suggests that a single TFIID species exists, that it binds to a wide range of TATA like sequences, and that, even in the absence of a detectable TATA box, TFIID may still be essential for RNA polymerase II mediated transcription. Moreover the finding that TFIID protects sequences from -35 to +40 from DNAase I digestion suggests that additional

sequences may play a role in TFIID binding. However, as other data show functional differences between TATA sequences (Simon et al., 1988), the significance of the precise TATA sequence remains controversial.

The ATF/CRE motif at -75 (TGTCGTCA) is not a perfect match to the consensus sequence for this element, TGACGTCA. Again, the significance of this deviation from the consensus is not clear. Non-consensus binding sites have proved to be effective in both baseline and cAMP stimulated transcription (Bokar et al., 1988) and ATF/CRE motifs with an A to T transversion at this position are present in the adenovirus E3 and E4 promoters and also in the heat shock protein 70 promoter (Lee et al., 1987). The TGTCGTCA motif was shown to bind proteins of the ATF/CRE family in gel retardation assay and competition experiments (figures 4.13, 4.14 and 4.15). The presence and binding activity of this site raised the possibility that the expression of orf62 might be responsive to stimulation by cAMP and the 289 amino acid product of the adenovirus E1A gene. Attempts to analyse the effect of altered cAMP levels on gene expression failed. It is relevant, however, that monomers of the ATF/CRE motif appear to mediate only small alterations in the levels of gene expression in response to cAMP and thus any effect on orf62 expression would be predicted to be relatively small. Moreover, it is likely that only certain members of the ATF/CRE family mediate this effect (J. Nevins pers. comm.) and until all of these proteins have been defined, and their individual binding specificities allocated, the precise function and relevance of this site should be regarded as preliminary. Thus the significance of this, potentially important, regulatory mechanism remains unclear.

Binding of proteins to the CCAAT box consensus was also demonstrated using both a fragment containing the CCAAT motif alone and one containing both CCAAT and ATF/CRE sites. As mentioned (section 1.5.2.3.), the family of CCAAT binding proteins appears to be even more diverse in terms of structure and function than the ATF/CRE family. Again for many of these proteins definition of the precise cis-acting sequences with which they interact and their potential for modification by post-translational mechanisms awaits cloning, sequencing and expression of the genes. Thus

little information can be deduced as to the significance of the CCAAT motif at -116, beyond its general and established function as a cis-acting element involved in transcriptional activation.

The use of fragment A which encodes both protein binding sites, along with competitor oligonucleotides allowed direct comparisons of the amounts of protein binding to these two sites in the orf62 promoter. This led to two observations. First, the CCAAT motif appeared to be responsible for a much higher degree of binding than the ATF/CRE binding site. Second, binding to the CCAAT box resulted in an apparent increase in binding to the ATF/CRE site. It is known that DNA binding proteins can interact to enhance the interaction of factors with adjacent binding sites. Data exists to show that this occurs in the binding of Oct-1 and Oct-2 to two different sites in the immunoglobulin heavy chain enhancer (Kelmer et al., 1989), binding of oestrogen receptors to two imperfect oestrogen responsive DNA elements, and for binding of the progesterone receptor to two adjacent progesterone response elements (Martinez and Wahli, and Tsai et al., abstracts of papers presented at the Cold Spring Harbour Meeting on Cancer Cells, Regulation of Eukaryotic mRNA Transcription, Sept. 6 to Sept 10, 1989, ppl42 and 212). These instances are not directly pertinent to the promoter of orf62. They all involve interactions between members of one protein species and binding sites that are separated by only a few bases. Thus, whether the effect seen in figures 4.11 and 4.12 represents a genuine example of different proteins interacting to modify binding, by a direct or indirect mechanism, or is merely an artifact of the assay system, is not clear. However, the fact that the the centres of both motifs are separated by 41 bases, an almost integral number of turns of B-form α-helix lends some, very circumstantial, support to the notion that a genuine interaction might take place, and suggests a way in which its importance might be tested.

The response of the plasmid pl40Δ131 to the 13S product of the adenovirus E1A gene was not unexpected, given the range of promoters stimulated by this protein (section 1.5.2.4.). However, this experiment was not primarily

designed to demonstrate the responsiveness of orf62 expression to E1A, but rather to provide functional corroboration for the protein binding data which suggested that the promoter sequence contained an ATF/CRE motif. In this respect it was only partially successful as recent data have shown that all three elements of the promoter can mediate transcriptional stimulation by the 13S product (section 1.5.2.4.). The available evidence tends to argue against mediation of 13S stimulation by the orf62 TATA box (Simon et al., 1988). However, discrimation between the ATF/CRE motif and the CCAAT box will await the availability of the functional and precise protein binding characteristics of the members of both families of proteins.

Thus the VZV orf62 promoter appears to consist of 3 elements, a TATA box, a ATF/CRE binding and a CCAAT motif. In overall structure this is very similar to the general arrangement found upstream of numerous other genes both viral and cellular. For example the beta-globin promoter comprises a TATA box (-26 to -30), a CCAAT box (-72 to -77) and a beta-globin specific element (-85 to -95) (Myres et al., 1986) and the adenovirus E3 promoter consists of a TATA box (-25 to -30), an ATF site (-54 to -61), an AP-1 site (-87 to -93) and a CCAAT box (-172 to -176) (Hurst and Jones, 1986). Thus the orf62 promoter, like those described above, falls into the pattern detailed in section 1.5.1., having a TATA box and one or more UASs within approximately 200 bp of the mRNA start site. It is noteworthy that both ATF/CRE and CCAAT motifs are common to many promoters and thus the proteins are, presumably, distributed amongst many tissues, so no particular tissue tropism for orf62 expression is suggested by the promoter sequences.

5.1.3. Vmw65 Responsive Region

The feature that distinguishes the regulatory sequences of α-herpes viruses from those of other viruses and from most other cellular promoters is the responsiveness of their IE genes to the HSV-1 transactivating protein Vmw65. Data both from functional analyses and protein binding assays presented here demonstrate that this is also true for the expression of VZV orf62. The data explain the findings of

Felsner et al., (1988) who characterised a cell line stably transformed with orf62. These workers showed that orf62 transcripts and protein product could only be detected in the presence of superinfecting HSV-1. The transcriptional stimulation was unaffected by the presence of cycloheximide during infection suggesting the involvement of a virion component. Felsner et al. failed to locate the 5' end of the mRNA and therefore assumed that, like HSV-1, the TATA box was situated around -350 relative to the first AUG of orf62. Thus their allocations of important cis-acting elements involved in the regulation of gene expression were not accurate.

The Vmw65 responsive region, -131 to -410, contains two motifs relevant to Vmw65 responsiveness, an octamer/TAATGARAT motif, centered at -256, and an octamer motif centered at -343. Only the octamer/TAATGARAT motif was able to induce efficient formation of a tertiary complex and, in common with the HSV-1 model, it was presumed to mediate Vmw65 responsiveness. The function, if any, of the octamer motif is not clear.

The demonstration that one of the cellular proteins involved in Vmw65 responsiveness is Oct-1 (Gerster and Roeder, 1988), led to the reassessment of the TAATGARAT motifs present in the upstream regions of the IE genes of HSV-1 and HSV-2 (apRhys et al., 1989). Several groups showed that TAATGARAT elements could be subdivided into two groups by the presence or absence of the residues ATGC immediately upstream (O'Hare and Goding, 1988; Gerster and Roeder, 1988; apRhys et al., 1989). The presence of these residues forms a 7 out of 8 match for the octamer motif while leaving the TAATGARAT element unaffected (ATGCTAATGARAT). In addition apRhys et al., (1989) suggested that all elements were present in the positive orientation with respect to the mRNA start site, by allocating those in the intergenic region between IE genes 3 and 4/5 to one or the other gene. The significance of the combination of the octamer and TAATGARAT motifs is not clear, as single octamer sequences alone do not appear to mediate Vmw65 responsiveness. However, evidence exists that the combined motif binds Oct-1 with higher affinity than TAATGARAT alone in the absence of Vmw65 (Baumruker et al.,

1988). Thus, the octamer motif might act to localise the cellular protein to the viral IE genes making the interaction with Vmw65 and the other cellular protein(s) at the TAATGARAT motif more efficient. Interestingly the only IE genes whose regulatory sequences contain simple TAATGARAT motifs and no combined elements are the IE-3 genes of HSV-1 and HSV-2. These regulatory sequences include an enhancer, and their transcription is least affected by the absence of Vmw65 (Ace et al., 1989). This suggests that they are less dependent on stimulation by the virion protein, and thus mechanisms that improve the efficiency of Vmw65 mediated transactivation might be of less importance.

The Vmw65 responsive motif of orf62 falls precisely into the pattern of the HSV IE genes, in that, lacking an enhancer, it contains an octamer/TAATGARAT motif and the whole motif is oriented in the positive sense with respect to the mRNA start site.

5.1.4. Sequences Further Upstream

The fourth region of potential interest in the regulation of orf62 transcription lies upstream of -410. Only sequences from -410 to -783 were analysed by gel retardation while functional analysis was performed on sequences from -410 to -1143. Protein binding assays consistently revealed a retarded band of low intensity with sequences between -668 and -783. Functional assays showed a small overall increase in both baseline and Vmw65 stimulated activity when plasmids containing this region were compared with those containing deleted sequences.

The region between -410 and -1143 contains two noteworthy sequence elements. One is the origin of DNA replication, and the other a set of 27bp direct repeats known as reiteration R4 (Davison and Scott, 1986). The reiterations contain 12 potential binding sites for the cellular transcription factor Sp1, which is not detected with the gel retardation protocol used (C. M. Preston, pers. comm.). These could be responsible for the increase in baseline expression seen in plasmids that incorporate this sequence, though in the SV40 system increased distance between the TATA box and a series of six Sp1 sites from 20

to 50 bp reduced expression by 50-fold (Takahashi et al., 1986). This result brings the importance of an array of Spl sites 455 bp upstream of the TATA box into question. Origins of DNA replication are known to be essential for the full expression of HSV L genes (Johnson et al., 1986) but have never been implicated in the control of IE genes. However, given the considerable potential for secondary structure resulting from their sequence, it is conceivable that they could affect transcription in vivo, under certain circumstances.

5.2. Analysis of orf10

The observation that the regulatory sequences of VZV orf62 are stimulated by the HSV-1 protein Vmw65 led to obvious interest in VZV orf10, whose predicted amino acid sequence shows 35% conservation with that of Vmw65 (Dalrymple et al., 1985; figure 4.18). Functional and protein binding analysis of this orf, presented in this thesis, revealed that it could neither stimulate transcription from a variety of promoters containing TAATGARAT elements, nor could it form complexes in gel retardation assays with cellular proteins and DNA fragments containing TAATGARAT elements. Thus the function of the product of orf10 in the VZV lifecycle appears to be confined to a structural role, equivalent to that of Vmw65, in the virion tegument. The sequences conserved between orf10 and UL48 may therefore provide an indication of residues important in virion structure and, by exclusion, protein-protein interactions important in transactivation.

One caveat must be noted with regard to these and other experiments in which negative results are derived from the analysis, in isolation, of the functions or products of DNA sequences. The possibility cannot be excluded that mutations accruing during the process of cloning or during subsequent manipulations might be responsible for the absence of activity. This pertains to the data on orf10 function, particularly those relating to protein-protein interactions. The possibility that mutation(s) in orf10 affected the results of binding assays was addressed by the

use of two independent clones of pORF10 in these experiments, but not by sequencing of the clones used. The expression of the VZV TK gene in vitro (Appendix 1) provides a salutary lesson in this regard. The absence of a recognised activating region from the sequence of orf10 supports the evidence from functional assays, making it unlikely that the inactivity as a transactivator results from mutations incorporated during manipulations. However, it remains possible that its ability to form a DNA binding complex with Oct-1 was affected by mutations accruing during its analysis.

The presence of orf10 in the VZV genome with its apparent homology to the structural but not the transactivating roles of Vmw65 raises the question of the mechanism(s) by which these proteins diverged and the selective pressures that resulted in this divergence. Two possible routes of evolution are, acquisition of the ability to transactivate by an orf 10-like precursor or loss of this ability by a Vmw65-like progenitor. The salient point in any progression between the two proteins is that two distinct requirements must be fulfilled in order for Vmw65 to transactivate, these are, complex formation with cellular proteins, and stimulation of transcription. As far as is known neither of these two aspects of Vmw65's activity has a role that is independent from the other. This does not present a problem in the transition from a transactivator to a non-transactivator, as loss of one function releases selective pressure on the other, resulting in its eventual loss. A model for evolution in the other direction, ie. to a transactivator, could be explained by one of three mechanisms. First, both functions could be acquired simultaneously. Second, either complex formation with cellular proteins or the interaction with transcriptional complexes could have or have had a role distinct from the other. Third, one of these functions could have been acquired or retained in the absence of selective pressures. The first and last of these options will be excluded from further discussion as no logical mechanism for them can be proposed. Thus either orf10 developed from a Vmw65-like protein whose transactivating function was lost, or a role for complex formation with cellular factors or for the

acidic activating region exists in isolation. The only absolute resolution to this problem would come from the discovery of an α-herpesvirus encoding a Vmw65 homologue with either the acidic activating region or the ability to form complexes with cellular proteins. In the absence of such a virus further analysis represents pure speculation. Intuitively one would argue against evolution away from a sophisticated and apparently advantageous (Ace et al., 1989) mechanism for the stimulation of IE gene expression, though the differences between HSV and VZV biology make this possible. A role for complex formation in the absence of transactivation could be related to the role of TAATGARAT sequences in the absence of Vmw65 as they may function in VZV and PRV. As will be argued below (section 5.3.2.) this may relate to the mechanism, by which α-herpesvirus IE genes are defined. A role for the acidic domain in the absence of complex formation is suggested by the findings of Sadowski et al., (1988) who showed that the presence of a strong activating region, in the absence of its binding motif can act, non-specifically, to reduce transcription. The transactivati ng domain of Vmw65 might originally have acted as an immediate inhibitor of cellular transcription. An attempt could be made to distinguish between these two possibilities by constructing a virus analagous to in1814 but abolishing the transactivating function on Vmw65 by deleting its activating region rather than by inhibiting complex formation.

5.3. Functional Significance of orf62 Regulatory Sequences

Considerable differences exist between the regulatory regions of the major IE genes of VZV, PRV and HSV. PRV is the least well defined and perhaps the most complex of these elements. It consists of a promoter, which contains a TATA box, a Sp1 site and an inverted CCAAT box, and an enhancer-like sequence. The enhancer contains six repeated 15bp elements similar to those present in the IE gene enhancers of HCMV and MCMV which are B-herpes viruses. In addition, inspection of the sequence in the light of the

importance of the octamer motif in Vmw65 responsiveness reveals octamer sequences centred at -250, -150 and -384. The latter two of these can be extended to give the sequences ATGCAAAGCAGAC and ATGCAAATCAGAG respectively which might be predicted to function as Vmw65 responsive elements. HSV IE gene 3 has a well defined arrangement of a TATA box and Spl sites forming the promoter, an enhancer sequence between -174 and -332 and TAATGARAT elements centred at -174 and -332 (Bzik and Preston, 1986). In contrast to the complex nature of these sequences orf62 has the simple arrangement described above (figure 4.5.).

Thus the expression of the IE genes of HSV-1, HSV-2, VZV and PRV are all stimulated by the HSV-1 virion factor, Vmw65, but neither VZV nor PRV apparently encode an active homologue. This raises the question of why the cis-acting elements that mediate the response are retained in the regulatory sequences of the VZV and PRV IE genes. The conservation of responsiveness to Vmw65 in PRV and VZV could be fortuitous and provide no selective advantage to either virus. This possibility cannot be excluded by the results presented here or elsewhere, however, it remains an explanation of last resort. Two overall mechanisms exist that would explain the conservation of this function in terms of its advantage to the virus. First, it could relate to the well defined mechanism of TAATGARAT controlled, Vmw65 mediated transcriptional stimulation. Second, it might imply the existence of a mechanism, separate from Vmw65 mediated transactivation, resulting from the interaction of Oct-1 or other octamer binding proteins with TAATGARAT.

5.3.1. A Cellular Homologue of Vmw65?

The first of these alternatives is basically an argument for another factor acting as a functional homologue of Vmw65, be it a cellular protein or another constituent of the VZV or PRV virions. The finding that PRV fails to stimulate the expression of HSV IE genes in the presence of cycloheximide (Campbell et al., 1987) and the absence of another sequence showing similarity to Vmw65 from the VZV genome (Davison and Scott, 1986), argues against the

presence of an alternative virion component acting in the same way as Vmw65. There is evidence to support the concept of a cellular factor that acts as a functional homologue of Vmw65. Efficient Vmw65 mediated transactivation depends on the formation of a multiprotein complex incorporating Vmw65, Oct-1 and one or more other cellular factors, all binding specifically to TAATGARAT (Section 1.4.2.). The salient feature of the formation of this complex is that, in the absence of Vmw65, Oct-1 alone binds to TAATGARAT (Gerster et al., 1988; Stern et al., 1989). Only with the addition of Vmw65 does recruitment of other cellular factor(s) take place. The presence of a cellular mechanism that facilitates the interaction of Vmw65 with Oct-1 suggests that this mechanism developed to allow the efficient interaction of Oct-1 with a cellular factor which functions as a Vmw65 homologue. This protein, if it exists, must be expressed in either a tissue or cell cycle specific fashion or have different binding constraints from Vmw65, as deletion of TAATGARAT elements from the HSV IE-3 or IE-4/5 promoters cause no reduction in transcription in the absence of Vmw65 (Preston, et al., 1984; Bzik and Preston, 1986). Stimulation of transcription by the octamer motif in the absence of Vmw65 appears to occur in the cell cycle dependent regulation of the H2b gene. In this system an octamer sequence, which can be extended to an octamer/TAATGARAT consensus (LaBella et al., 1988), is responsible for cell cycle co-ordination of the expression of the H2b gene. This could signify the existence of a cell cycle specific Vmw65 homologue, though a separate, cell cycle specific, transactivating, octamer binding protein could also explain the findings. An alternative explanation for the existence of this "interaction factor" is that it acts, more generally, to facilitate protein-protein interactions with DNA binding proteins other than Oct-1. In this regard it is relevant that the homeodomain of Oct-1 is necessary and sufficient to mediate the interaction with Vmw65 (Stern et al., 1989). The cellular factor(s) recruited by Vmw65 could normally function in interactions between homeodomain containing proteins other than Oct-1 and transcriptionally active factors equivalent to Vmw65. Thus, the activity of the VZV and PRV Vmw65 responsiveness

elements in recruiting a Vmw65 homologue *in vivo* and thus stimulating IE gene expression, remains unproven.

5.3.2. An Additional Role for TAATGARAT?

A second possible explanation for the Vmw65 responsiveness of the VZV and PRV IE genes is that the conservation of the Vmw65 response element, TAATGARAT, results from its role in a mechanism separate from Vmw65 mediated transcriptional activation. One obvious function for this motif would be in providing the minimal signal necessary to distinguish α-herpesvirus IE genes from the other temporal classes. This mechanism has not been defined in any of the α-herpesviruses, though two simple models can be proposed to explain it. First, promoter strength alone could result in the expression of IE genes swamping that of other viral genes and preventing their detection prior to the active down regulation of IE expression and the stimulation of E gene expression by the IE gene products. This view gains some credence from the finding that some of the most potent regulatory sequences known are found upstream of viral IE genes. However, the relative weakness of the orf62 regulatory sequences in the absence of Vmw65, argues against this possibility. The second model involves a specific signal which denotes IE genes. This signal could act either to allow viral or cellular factors to stimulate the expression of IE genes or, conversely, to prevent their suppression. Evidence that TAATGARAT represented this minimal essential signal came from data which showed that this motif alone converted an IE promoter-CAT, plasmid construct, to IE type regulation in short term transfection assays (Gaffney *et al.*, 1985). However, a role for Vmw65 transactivation in this effect is ruled out by its absence from PRV and VZV (Campbell *et al.*, 1987; this thesis), and by the regulation of IE genes in the HSV mutant *in*1814 (Ace *et al.*, 1989). Thus, by elimination, a cellular mechanism that either actively stimulates expression, or specifically relieves suppression, of IE regulatory sequences seems the most likely mechanism for defining α-herpes virus IE genes. Given the conservation of the TAATGARAT motif in α-herpesvirus IE gene regulatory sequences, the interaction

of Oct-1 with this element represents a good candidate for a mechanism to define IE genes. However, any mechanism proposed that suggests a role for Oct-1 in activating IE genes has to explain the general inability of this factor to stimulate transcription from TATA box dependent promoters in the absence of Vmw65 (section 1.4.3.). Some evidence that such a mechanism exists comes from an observation that binding of Oct-1 to TAATGARAT induces an alteration in DNA conformation when compared with binding of purified preparations of the same protein to octamer sequences. This alteration was detected as a different mobility of complexes binding to the two motifs in gel retardation assays (apRhys et al., 1989), and might suggest that a conformational change in DNA structure results in IE regulatory sequences being made available to transcriptional complexes. Evidence against this concept comes from a series of HSV-2 mutants in which all of the TAATGARAT elements were deleted from the upstream region of IE-3 (Smith et al., 1989). Growth of these viruses under cycloheximide block resulted in reduced but detectable IE-3 mRNA synthesis. However, the authors also constructed a virus which lacked all of the conventionally recognised regulatory sequences of IE-3, including the TATA box. This mutant also produced detectable IE-3 mRNA under cycloheximide block, thus the significance of the results is not clear.

5.4. A Connection Between IE Gene Regulation and Virus Biology?

VZV remains something of an enigma. It is a very successful and well adapted human parasite with no obvious difficulty in propagation or infectivity in its natural host, yet in tissue culture the only efficient technique to initiate infections is by the use of infected cells. The VZV system also lacks a convenient animal model that mirrors viral pathogenesis in humans. Does the regulation of the IE genes have any bearing on this? Evidence that it might comes from the phenotypes of mutant viruses in which the expression of functional IE gene products is curtailed either by mutations in their coding sequences (Harrison et al., 1977; Jones and Shenk, 1979; Shenk et al., 1979; Stow

and Stow, 1986; Everett, 1989) or by down regulation of their expression (Ace *et al.*, 1989). In such viruses the absence or reduced expression of IE proteins results in defective growth both *in vitro* and *in vivo*. The growth defect can be overcome by high multiplicities of infection. It is obviously impossible to extrapolate directly between HSV and adenovirus mutants and wild type VZV but if the use of infected cells is viewed as generating localised high multiplicities of infection then some resemblance is present. Attempts were made to rescue this proposed defect in VZV IE gene regulation by coinfection of UV irradiated *ts*K. Controls for this experiment showed that the *in vitro* phenotype of *in*1814 could be rescued using this technique (Ace *et al*, 1989) but the failure to produce cell free VZV precluded a definitive answer.

5.5. Future Prospects

The overall aims in future work on VZV are to increase the understanding of this important but ill understood pathogen. This will inevitably continue to be hindered by the lack of high titre stocks of cell free virus and of a good animal model system. Thus, the best hope for significant advances appears to be the analysis of individual orfs in isolation. This process will be facilitated by the presence of homologues of many VZV genes in the HSV genome. The drawback of this high degree of input from the HSV system is that it results in a tendency to view VZV as an inferior version of HSV-1.

The analysis of the IE genes of VZV is held back by the lack of basic information. The only data available to date regarding the identities of these genes are sequence homology with HSV genes and the molecular weights of bands determined by SDS-PAGE, from cells infected with VZV under cycloheximide block. One method by which these genes might be identified would be to map the mRNA initiation sites of candidates and analyse their regulatory sequences *in vitro*, though absolute confirmation of the identities of the VZV IE genes will await antisera directed against individual polypeptides.

With respect to this project, work could be continued

to analyse the interaction between the promoter motifs, perhaps by introducing five and ten base insertions and deletions between them. In addition the site of the E1A responsiveness could be located within the promoter by further deletion analysis and the replacement of the VZV TATA box with that of SV40 which is known to be unresponsive to E1A. Finally the availability of cell free VZV would allow the effect of altered IE gene regulation on virus titre to be tested.

APPENDIX 1

Expression of the VZV Pyrimidine Deoxynucleoside Kinase Gene

As detailed previously (section 1.3.5.1.) orf36 encodes the VZV dPyK gene. It was decided to express the product of this orf using the plasmid pKK233-2 (Amman and Brosius, 1985) which was designed to allow the expression of unfused eukaryotic proteins in E.coli. This system potentially allows easy manipulation of the primary sequence enabling correlations to be drawn between enzymatic activity and specific amino acid sequences. Prokaryotic systems are also convenient for the production of large quantities of proteins and thus have the potential to allow analysis of tertiary structure. pKK233-2 contains the highly expressed trc promoter (a trp-lac fusion promoter with the consensus 17bp spacing between the trp35 region and the lac UV5-10 region), the lacZ ribosome binding site and ATG initiation codon. The ATG start codon is located 8 bases downstream from the ribosome binding site within a unique NcoI site (Amman and Brosius, 1985). This site can be cleaved to generate a 5' overhang compatible with that of the restriction endonuclease AflIII which spans the initiation codon of VZV orf36.

A 1103bp EcoRI-SnaBI fragment containing orf36 was excised from the VZV clone Sst h (kindly provided by Dr. A. Davison) and inserted between the HincII and EcoRI sites of pUC9 (Figure A1. 1.). A partial digest of this plasmid with AflIII was then carried out followed by complete digestion with HindIII. This produced a 1043bp fragment which was inserted between the NcoI and HindIII sites of pKK233-2, to form plasmid pTMORF36. Several independent clones of this plasmid were isolated, and two were grown up into large scale preparations (pTMORF36.1 and pTMORF36.2).

To analyse the proteins produced by this construct, E.coli W3110 cells were transformed with pTMORF36.1 and 2 and grown in the presence or absence of IPTG. Lysates of these cells were separated using a 12% SDS-PAGE gel and stained with coomassie brilliant blue (figure A1.2). Lanes 1 and 2 show untransformed W3110 in the presence and absence

of IPTG. Lanes 3 to 6 show cells transformed with pTMORF36.1 and 2 in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of IPTG. Two novel bands were observed in lanes 4 and 6. These are labelled *B* and *A* and their approximate molecular weights are 15,000 and 40,000 respectively. A slight increase in intensity of a band, labelled X, is also observed in lanes 4 and 6. It is not clear whether this represents an induced species or merely overloading of these tracks. If it does represent an induced species then its source cannot be explained. Lanes 7 and 8 show the lysate of W3110 cells transformed with the plasmid pCI15 (Amman and Brosius, 1985) which contains an N-terminal deletion mutant of the cI repressor in the same background as pTMORF36.1 and pTMORF36.2. This plasmid is known to express a protein of molecular weight 26,000 (labelled C). The predicted molecular weight of the VZV TK is 37,814 which corresponds approximately to band A. It is not clear what band B represents though it is possible that it results from proteolytic cleavage of the species present in band A.

Experiments were then undertaken to compare dPyK activity in extracts of W3110 cells transformed with pTMORF36.1, pTMORF36.2, pKK233-2 and the plasmid pTK1 which contains the HSV TK gene (table A1.1). An initial experiment used the assay system optimised for the HSV TK (table A1.1 section A). This assay gave background values in extracts of cells transformed with pTMORF36.1, pTMORF36.2 and pKK233-2 but an obvious increase in activity in cells transformed with pTK1. Further experiments, used protocols previously described for the detection of activity from the VZV dPyK (Doberson et al., 1976; Shiraki et al., 1985). Again, no difference in activity could be detected when extracts from transformed and untransformed cells or from cells incubated in the presence or absence of IPTG were compared. Thus, despite the production of considerable amounts of proteins, no novel activity was detectable in cells expressing VZV orf36.

Since this work was completed, both published and unpublished reports have provided an explanation for these apparent contradictions. Recent comparisons of dPyK sequences from wild type VZV strains and those of a series

of acyclovir resistant VZV mutants revealed one mutation (T->C at +853, resulting in a Leu to Ser substitution at residue 288) that was unique to the Dumas strain of VZV. This mutation distinguished the dPyK gene of the Dumas strain both from all other VZV strains sequenced (Sawyer et al., 1988) and from published TK sequences of other herpes viruses (Mittal and Field, 1989). The mutation falls within a region of high conservation amongst herpes virus TK sequences although it is not within the defined nucleotide or nucleotide/nucleoside binding sites (Ōsuka and Kit, 1984; Gentry, 1985; Darby et al., 1986) and is absent from the sequence of the marmoset herpes virus TK (Ōsuka and Kit, 1984). Recent unpublished evidence from P. Harrison (pers. comm.) have provided direct evidence that the Leu to Ser substitution at residue 288 is responsible for the inactivity of the enzyme. Site directed mutagenesis was carried out on pTMORF36.1 to convert the C residue to a T at position +853 thus returning the Ser to a Leu at residue 288. This manipulation resulted in a plasmid which unequivocally expressed TK activity under the conditions optimised for both HSV-1 and VZV enzymes (described above). Interestingly, as noted above, residue 288 does not fall within regions previously recognised as being essential for enzyme function suggesting that this residue may serve an role in the structural integrity of the protein, its ability to dimerise or its enzymatically active site or sites.

Figure A1.1. STRUCTURE AND CONSTRUCTION OF pTMORF36.

pTMORF36 is derived from the vector pKK233-2 (Amman and Brosius, 1985), andthe VZV clone Sst h. The shaded rectangle represents orf36, P_{trc} is a trp-lac fusion promoter and 5S represents the 5S rRNA gene and transcriptional and transcriptional terminators. Relevent restriction endonuclease sites are marked. The EcoRI-SnaBI fragment is 1103bp and the AflIII-HindIII fragment is 1035bp.

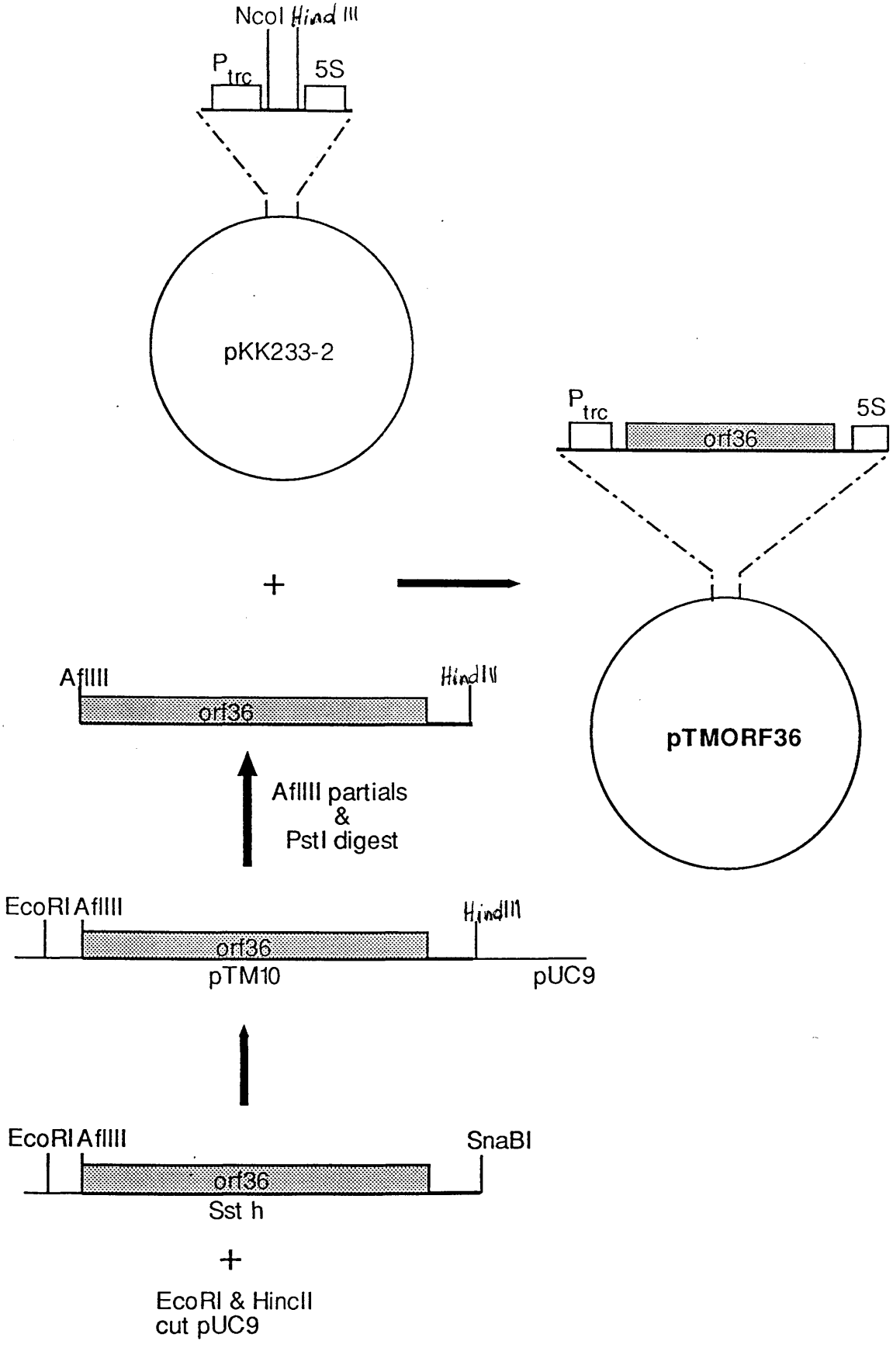

NcoI HindIII
Ptrc
5S
pKK233-2
+
Ptrc
orf36
5S
pTMORF36
AflIII
HindIII
orf36
AflIII partials
&
PstI digest
EcoRI AflIII
HindIII
orf36
pTM10
pUC9
EcoRI AflIII
SnaBI
orf36
Sst h
+
EcoRI & HincII
cut pUC9

Figure A1.2. EXPRESSION FROM PLASMID PTMORF36

Plasmids were expressed in E.coli W3110 cells. Lanes 1 and 2 show polypeptide synthesis from untransformed cells. Lanes 3-8 show expression from cells transformed with pTMORF36.1 (3 and 4), pTMORF36.2 (5 and 6) and pCI (7and 8). Cultures were incubated for four hours of 37^{o}C and IPTG was added in lanes 2, 4, 6 and 8 after two hours. Cells were lysed and separated by SDS-PAGE prior to staining with Coomassie Blue. The novel bands are labelled A, B, C and X, and are referred to in the text.

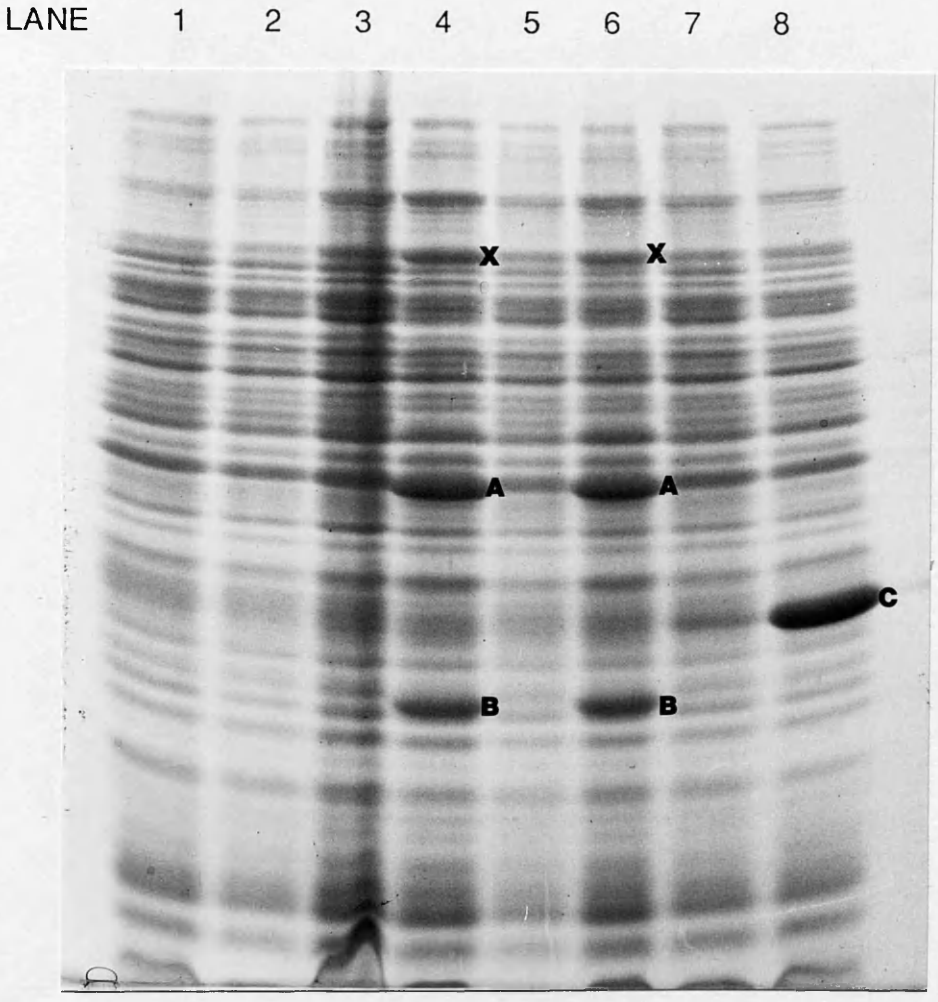

LANE 1 2 3 4 5 6 7 8
X
X
A
A
C
B
B

Table A.1.1.

TK assays, on lysates of W3110 cells, transformed with the plasmids indicated and grown in the presence (+) or absence (-) of IPTG were carried out, as described. In section A, results are expressed in counts per minute while in section B they are expressed in counts per minute x 10^{-3}. The assays in section B were performed in the presence and absence of dTTP.

Table - A1. 1. TK ASSAYS OF EXTRACTS OF CELLS TRANSFORMED WITH pTMORF36.

A). HSV TK ASSAY CONDITIONS.

IPTG 1mM	pTMORF36.1	pTMORF36.2	pKK233-2	pTK1
+	3900	3800	3700	39500
-	3100	3900	-	55900

B). VZV TK ASSAY CONDITIONS

counts x 10^{-3}

Plasmids	pCI	pTMORF36.1		pTMORF36.2		
IPTG 1mM	+	-	+	-	+	
pH7.5	25	22	8	42	21)	+TTP
pH8.0	107	90	19	130	96)	
pH7.5	119	177	122	204	174)	-TTP
pH8.0	162	33	129	147	138)	

APPENDIX 2

Purification of Recombinant Viruses Using B-galactosidase Expression as a Marker.

The aim of the experiments presented here was to express the putative protein kinase encoded by VZV orf66 in quantities sufficient for structural and functional analysis. To achieve this, it was decided to use the HSV-1 mutant tsK, which at non-permissive temperature, over-expresses its immediate early polypeptides (Preston, 1979; Section 1.3.2.1.). The aim was to insert orf66 with HSV-1 IE control sequences into the TK gene of tsK and select recombinant progeny using the nucleoside analogues bromodeoxycitidine (BCdR) or acyclovir. The plasmid A494*23, constructed by C. Preston (figure A2.1), is based on pAT153 and has the HSV BamHI p fragment, which encodes the HSV TK gene, inserted into a unique BamHI site. A 360bp fragment encoding the control sequences of the HSV-1 IE3 gene, with a unique XhoI site downstream, was inserted into an SstI site within the coding sequence of the TK gene.

The orf66 coding sequences were excised from the genomic clone Kpn j in two parts (figure A2.2). A 2140bp BamHI subfragment was inserted into pUC9 to form pTM3, and a 940bp SspI subfragment inserted into pUC9 to form pTM2. A 465bp AccI/SnaBI fragment was transferred from pTM3 to pTM2 between a blunt ended PstI site and a SnaBI site. XhoI linkers were inserted to flank orf66 forming pTM4. The XhoI fragment was inserted into the XhoI site of A494*23 to form pTM7.

This plasmid was co-transfected with tsK DNA and both BCdR and acyclovir used in attempts to select TK^- recombinants. Ten TK^- isolates were prepared but the presence of orf66 could not be detected when these were analysed by Southern hybridisation, suggesting that the viruses selected resulted from spontaneous muta tions in the TK gene rather than insertion of orf66. In order to circumvent this problem it was decided to produce a virus

encoding the B-galactosidase gene under the control of IE regulatory sequences and select recombinants by the presence or absence of B-galactosidase activity in the plaquing overlay. The presence of the enzyme can easily be detected by its action on the chromogenic substance, X-gal. The B-galactosidase gene and SV40 polyadenylation signals were excised from the plasmid pFJ3 as a 3737bp HindIII fragment. The fragment was inserted into the XhoI site of A494*23 (figure A2.2.) to form pTM8. pTM8 was co-transfected into BHK cells along with tsK DNA and recombinant viruses selected by their ability to metabolise X-gal. One recombinant, VT1, isolated in this way, was subjected to three rounds of plaque purification and grown in bulk to allow the preparation of viral DNA.

VT1 DNA was then co-transfected with pTM7 or A494*23, a negative control and recombinant viruses selected by their inability to metabolise X-gal. Ten virus stocks were obtained from transfections with pTM8 and four from transfections with A494*23. These were each subjected to four rounds of plaque purification. Proteins produced by these viruses and VT1 were analysed using SDS-PAGE following pulse labelling with [^{35}S]-methionine at non-permissive temperature. The protein profiles of viruses VTM7.1-10 were identical as were those of VTM23.1-4, thus, for ease of presentation a representative sample is shown (figure A2.3.). The proteins produced by VT1 under IE conditions are shown in lane 5 . The prominant band, marked B-gal, with a relative mobility of approximately 116,500, was assumed to be the product of the B-galactosidase gene. The proteins produced by VTM7.1 and VTM7.2 (lanes 3 and 4) lack this band and fail to show any novel band corresponding to the product of orf66 whose predicted molecular weight is 43,577. Lane 2 shows the proteins produced by VTM23.1. This lane again lacks the 116,500 product but this is replaced by a band running with an approximate relative molecular weight of 25,000. This may represent the carboxy-terminal portion of the HSV TK expressed under the control of the IE 3 promoter as the first ATG codon downstream of the IE-3 regulatory sequences occurs in the TK reading frame and has a good (6 out of 8) match to the Kozak consensus (Kozak, 1984).

The genotypes of the viruses were analysed by Southern hybridisation, using the plasmid pTK as a probe (figure A2.4). Virus stocks VTM7.1-10 (lanes 1-5 and 13-17) have a restriction pattern consistent with the insertion of the coding sequences of pTM7 into the TK gene (compare with lane 23). In addition, VTM1 and VTM23.1 also produced the expected patterns. This confirms that orf66 was inserted, apparently correctly, into the TK gene of tsK. In spite of this no protein was produced.

There are numerous possible stages at which a mutation could have arisen preventing the expression of orf66. The plasmid A494*23 has been used successfully for the expression of HSV orfs (J. Calder pers. comm.) and thus a fundamental defect in it is unlikely. It also seems unlikely that the problem has occurred at the stage of virus construction as all ten virus stocks appear to be identical both in terms of proteins produced and the fragment inserted. Thus the most likely reason for the failure of protein expression is the incorporation of mutations during the production of pTM7. This possibility was not investigated further.

Figure A2.1 CONSTRUCTION OF PLASMID A494*23

This plasmid was constructed from the vector pAT153. The BamHI p fragment, derived from the genomic clone is represented by a heavy line and the coding sequences of the HSV-1 TK gene by an open box. The 360bp fragment containing the HSV-1 IE gene 3 control sequences is represented by black shading. Relevant restriction endonuclease sites are marked.

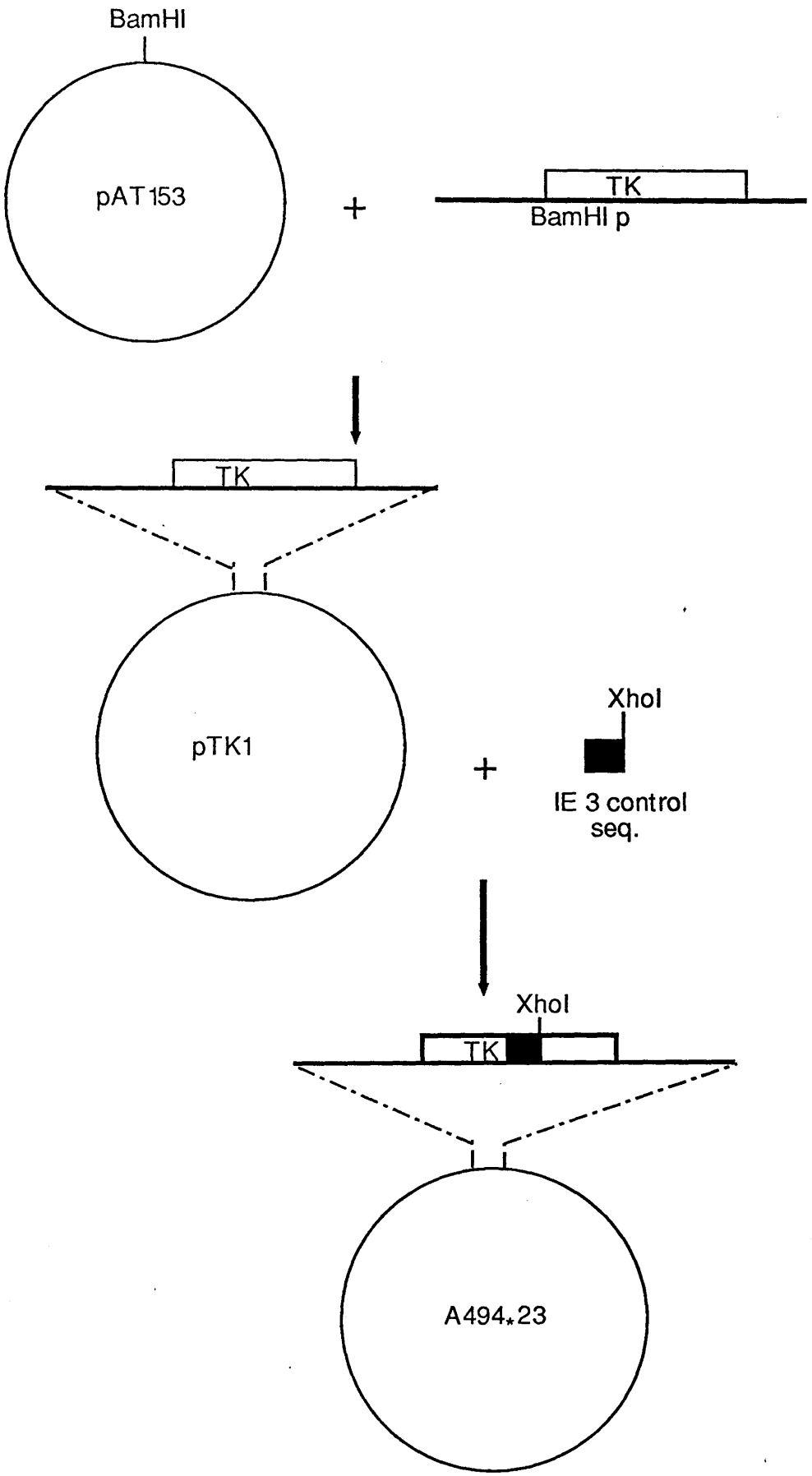

BamHI
pAT153
+
TK
BamHI p
TK
pTK1
+
XhoI
IE 3 control
seq.
XhoI
TK
A494*23

Figure A2.2. CONSTRUCTION OF PLASMIDS pTM8 AND pTMORF66

Both plasmids were derived from the plasmid A494*23 (figure A2.1.). The HSV-1 BamHI p fragment is represented by a heavy line, the coding sequences of the HSV TK gene by an open rectangle and the HSV-1 IE sequences by black shading. Both the E.coli B-galactosidase gene and VZV orf66 (shaded rectangles) were inserted into the XhoI site of A494*23 forming pTM8 and pTMORF66 respectively. Relevant restriction endonuclease sites are marked. The fragment marked B-gal is 3737bp in length and is derived from pFJ3. The fragment marked orf66 was derived from VZVKpn j. The complete orf was constructed from two overlapping fragments, pTM2 which contains a 940bp SspI fragment, and pTM3, 2140 BamHI fragment, both of which were inserted into the pUC9 vector. The sub-fragments were joined using a SnaBI site within the overlap. Constructs were checked using restriction enzyme analysis.

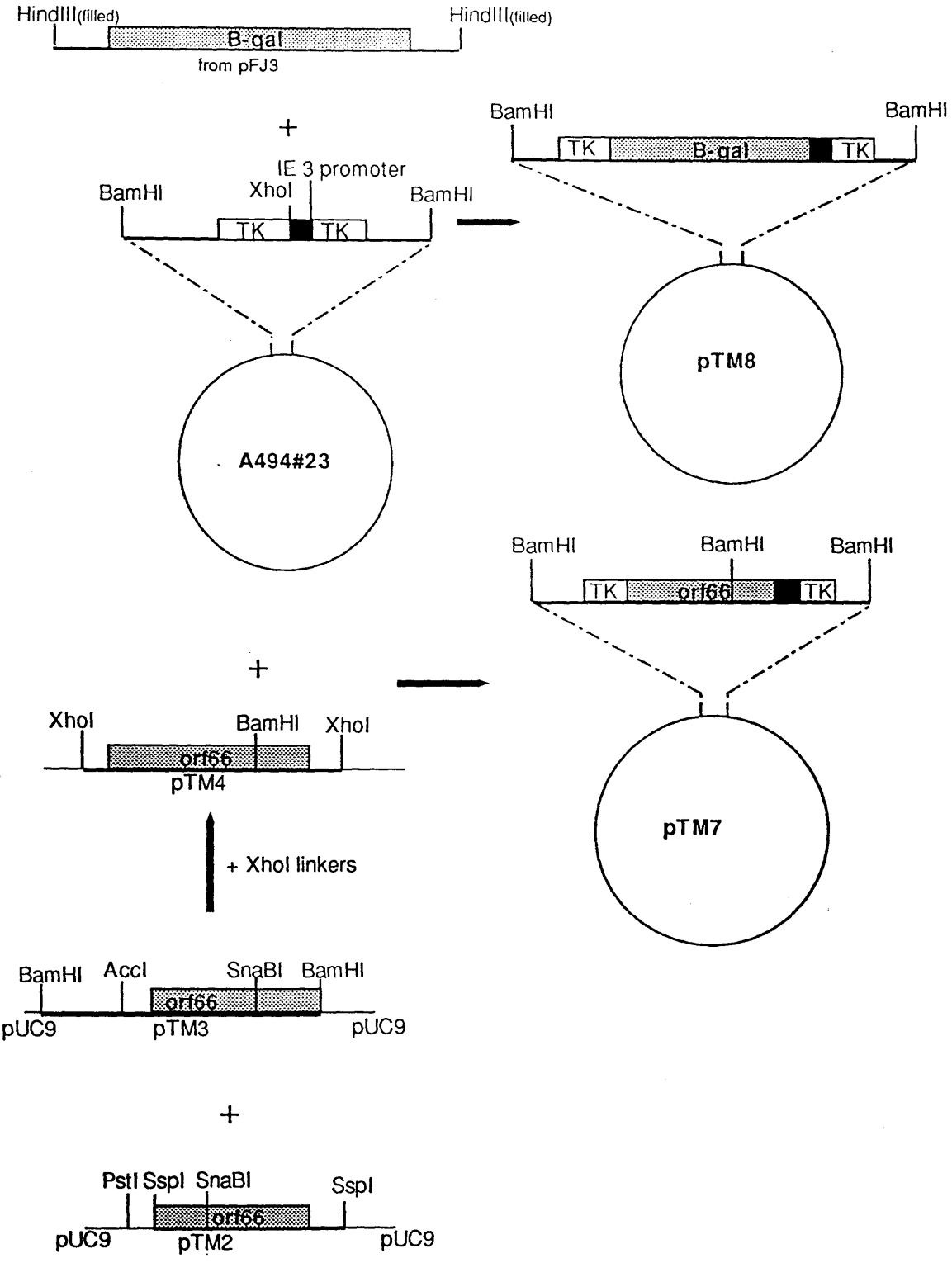

HindIII(filled)
B-gal
HindIII(filled)
from pFJ3
+
IE 3 promoter
BamHI
XhoI
BamHI
TK
TK
A494#23
BamHI
BamHI
TK
B-gal
TK
pTM8
BamHI
BamHI
BamHI
TK
orf66
TK
pTM7
+
XhoI
BamHI
XhoI
orf66
pTM4
+ XhoI linkers
BamHI
AccI
SnaBI
BamHI
orf66
pUC9
pTM3
pUC9
+
PstI
SspI
SnaBI
SspI
orf66
pUC9
pTM2
pUC9

Figure A2.3. ANALYSIS OF PROTEINS PRODUCED BY RECOMBINANT VIRUSES

Cells were mock infected (lane 1) or infected with VTM23.1, VTM7.1, VTM7,2 or VTM1 (lanes 2,3,4, and 5, respectively) in the presence of cycloheximide. Proteins were labelled with [^{35}S] methionine after removal of the cycloheximide by washing at 4h post infection. Proteins produced were separated by SDS-PAGE on a 12% gel and detected by autoradiography.

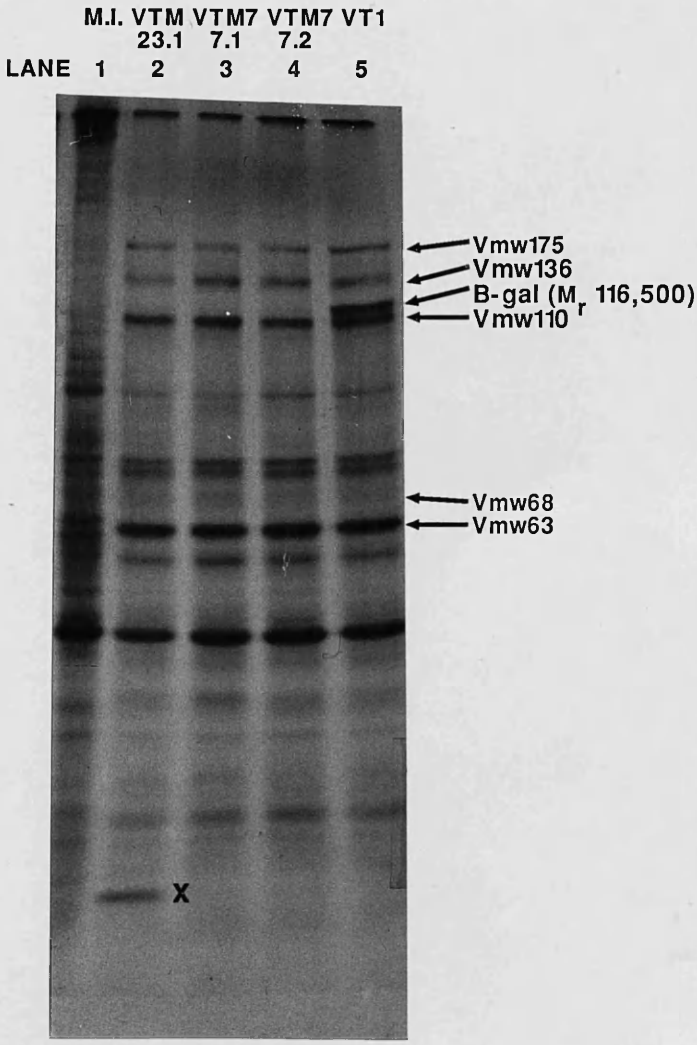
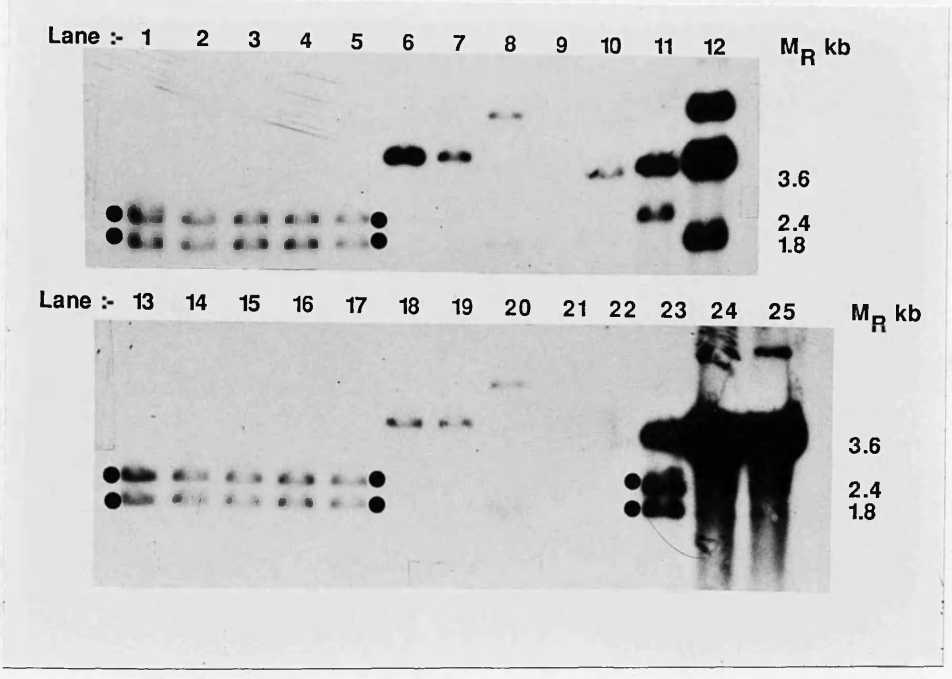

M.I.
VTM 23.1
VTM7 7.1
VTM7 7.2
VT1
LANE 1 2 3 4 5
Vmw175
Vmw136
B-gal (Mr 116,500)
Vmw110
Vmw68
Vmw63
X

Figure A2.4. ANALYSIS OF THE GENOMES OF RECOMBINANT VIRUSES

DNA was prepared from plaque purified isolates of recombinant viruses. The DNA was cleaved with BamHI and loaded anto a 1% agarose gel in the following pattern.

Lanes 1-5 and 13-17, VTM7.1-10 respectively.
Lanes 6, 7, 18 and 19, VTM23.1-4 respectively
Lanes 8 and 20, VTM1
Lanes 9 and 21, mock infected
Lanes 10 and 22, tsK
Lanes 11, 12, 23, 24, and 25 were marker tracks containing plasmid DNA. Lane 23 is BamHI-cleaved pTM7, generating 1.8kbp and 2.4kbp bands, marked (●) that identify the presence of orf66.

DNA fragments were separated, transferred to nitrocellulose and hybridised with a ^{32}P labelled probe derived from plasmid pTK which contains the sequence of the HSV-1 TK gene. The sizes of the fragments are as marked.


Lane :- 1 2 3 4 5 6 7 8 9 10 11 12
M_R kb
3.6
2.4
1.8
Lane :- 13 14 15 16 17 18 19 20 21 22 23 24 25
M_R kb
3.6
2.4
1.8

Appendix 3

Oligonucleotides Used in the Project

Primer Extension Oligonucleotide, single stranded, sequence taken from Davison and Scott, (1986), bases 109100 to 109127.

5'-GGGGTGTAGAGCGCTGCATCGGCGGCGT-3'

S1 Nuclease Mapping Oligonucleotide, single stranded, sequence taken from Davison and Scott, (1986), bases 109159 to 109238.

5'-GAGTTTTCCAAACGCTCTCGCAGCCTTCAAAGGATTGCGATTGCGGTGAGGGAGTT
CCAACAGTACTTAAAACG-3'

5' Untranslated Region of VZV orf62 as incorporated into pTM15cat, double stranded, sequence taken from Davison and Scott, (1986), bases 109159 to 109238.

5'-CACGTTTTAAGTACTGTTGAAACTCCCTCACCAACCGCAATCGCAATCCTTG-3'

Oligonucleotide 4 used in gel retardation assays, double stranded, sequence taken from Davison and Scott, (1986), bases 109344 to 109310.

5'-AGCTTCTTCCCGCCTCGAGTCTCGTCCAATCACTACCATCG-3'

Oligonucleotide 5 used in gel retardation assays, double stranded, sequence taken from Davison and Scott, (1986), bases 109,309 to 109264.

5'-TCTTATCATTAAGAATATTTACACGGTGACGACACGGGGAGGAAAG-3'

Oligonucleotide BS2wt, from Hurst and Jones, (1987), encodes the ATF motif from the adenovirus 5 E3 promoter. A kind gift of Dr. N. Jones.

5'-GGCGGCTTTCGTCACAGGGTGCGG-3'

Oligonucleotide BS2/4, fron Hurst and Jones, (1987), encodes a non-functional ATF concensus the mutation that differentiates it from BS2wt is underlined. A kind gift of

Dr. N. Jones.

5'-GGCGGCTTTCTTCACAGGGTGCGG-3'

Oligonucleotide BS3wt, from Hurst and Jones, (1987), encodes the AP1 motif from the adenovirus type 5 E3 promoter, Hurst and Jones, (1987). A kind gift of Dr. N. Jones.

5'-GAAGTTCAGATGACTTAACTCAG-3'

Octamer Oligonucleotide. The octamer motif (underlined) was derived from sequences upstream of the rearranged V_HDJ_H region of the murine gene (Grosscheld and Baltimore, 1985). A gift of Dr. C. M. Preston.

5'-AGCTTGCCTCATGAGTATGCAAATCATGTGCGACTG-3'

TAATGARAT Oligonucleotide. The TAATGARAT motif (underlined) was taken from the control sequences of the HSV-1 IE3 gene and inserted into an identical background as the octamer motif (above). A gift of Dr. C. M. Preston.

5'-AGCTTGCCTCATGAGTGCGGTAATGAGATGCGACTG-3'

HSV TK CCAAT Box Oligonucleotide, Preston et al., (1988). Double stranded oligonucleotide representing sequences -66 to -100 in the HSV-1 TK gene. A kind gift of Dr. C.M. Preston.

5'-AGCTTGCGTCTTGTCATTGGCGAATTCGAACACG-3'

Adenovirus 5 Major Late Promoter oligonucleotide Preston et al., (1988). Double stranded oligonucleotide representing sequences -44 to -88 in the adenovirus 2 major late promoter. A kind gift of Dr. C.M. Preston

5'-AGCTTGGTTTATAGGTGTAGGCCACGTGACCGGGTG-3'

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

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

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

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

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

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MATERIALS AND METHODS

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

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

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

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

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

Radiolabeling of viral polypeptides. For IE polypeptides, BHK cell monolayers were infected in the presence of 200 μg of CH per ml. After 4 h at 38.5°C, CH was washed from the plates and proteins were radiolabeled for 1 h with [^{35}S]methionine in the presence of 1 μg of actinomycin D per ml and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (46). For early and late polypeptides, BHK cell monolayers were infected and incubated at 37°C for 8 h, and proteins were radiolabeled for 1 h with [^{35}S]methionine and analyzed by SDS-PAGE.

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

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

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

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

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

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

RESULTS

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

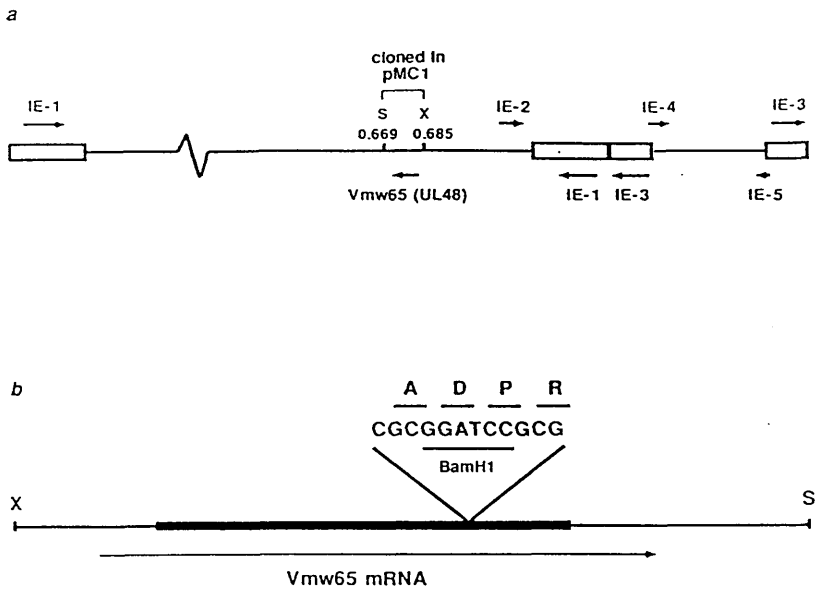

FIG. 1. (a) Structure of the HSV-1 genome showing the positions of the five IE genes (without introns) and Vmw65. The open boxes represent repeated sequences. (b) Structures of the insertion mutation in Vmw65 encoded on a *Sal*I(S)-*Xho*I(X) fragment in plasmid pMC1.in14.

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

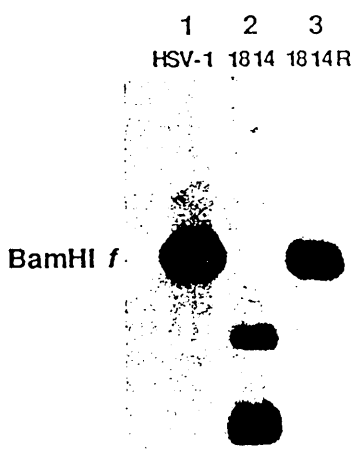

FIG. 2. Structure of the *in*1814 genome. Wt HSV-1 (lane 1), *in*1814 (lane 2), or 1814R (lane 3) DNA was cleaved with *Bam*HI, and the fragments were separated on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pMC17. The position of HSV-1 *Bam*HI *f* is indicated.

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

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

[a] ND, Not determined.

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

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

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

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

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

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

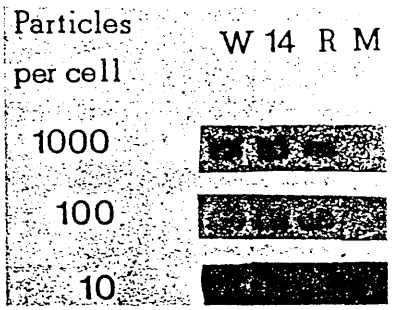

FIG. 3. DNA migration to the nucleus. DNA isolated from nuclei of cells infected with wt HSV-1 (W), *in*1814 (14), or 1814R (R) or mock infected (M), in the presence of CH, was cleaved with *Bam*HI, and the fragments were separated on an agarose gel, transferred to GeneScreen Plus membrane, and probed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

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

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

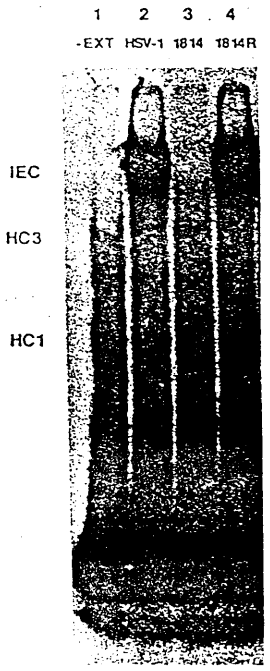

FIG. 5. IEC formation by Vmw65. A 74-bp DNA fragment containing the IE gene 4/5 TAATGAGAT sequence was incubated with HeLa cell nuclear extract alone (lane 1) or with virion extract (EXT) from wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and was analyzed by gel electrophoresis. The positions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

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

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

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

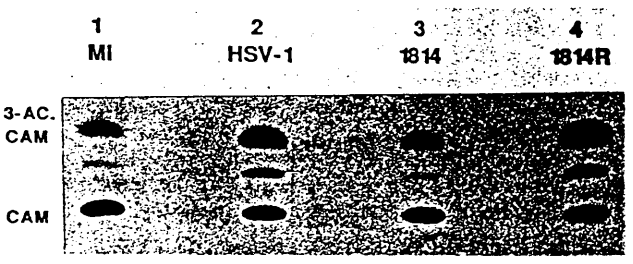

FIG. 6. Transinduction of IE transcription. Cells were transfected with pIE3CAT and mock infected (lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4). Chloramphenicol acetyltransferase assays were carried out on cytoplasmic cell extracts. The positions of chloramphenicol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

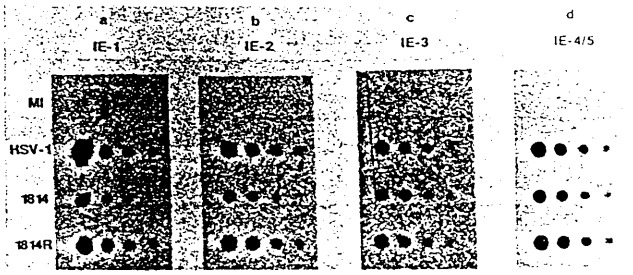

FIG. 7. Production of IE RNA. Cells were mock infected (MI; row 1) or infected with wt HSV-1 (row 2), *in*1814 (row 3), or 1814R (row 4) in the presence of CH. RNA was prepared after 4 h and applied to nitrocellulose filters in four sequential dilutions (3 μg, 1 μg, 0.3 μg, and 0.1 μg). Filters were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

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

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

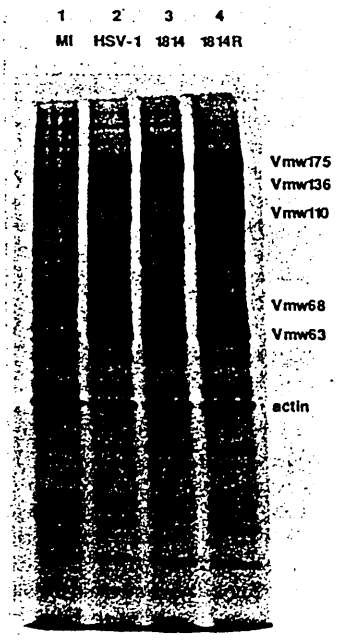

FIG. 8. IE polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4) in the presence of CH. Proteins were labeled with [^{35}S]methionine after removal of CH by washing at 4 h postinfection. The positions of viral IE polypeptides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

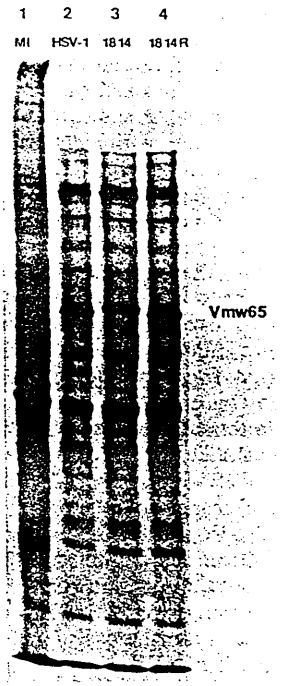

FIG. 9. Late polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and proteins were labeled with [^{35}S]methionine at 8 h postinfection. The position of Vmw65 is indicated.

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

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

Thus, upon infection of BHK cells with 1,000 particles of *in*1814 per cell in the presence of CH, the level of expression of IE genes 1, 2, 4, and presumably 5 is reduced, but under normal conditions infection procedes to the late stage, suggesting that there is no overall consequence of reduced IE gene expression. From the high particle/PFU ratio, however, it appears that the growth of *in*1814 is inefficient when cells are infected with 1 virus particle per cell. To investigate whether the incapacity of *in*1814 at low MOI is reflected in reduced gene expression, the synthesis of TK, an early enzyme that can be detected with high sensitivity, was examined. BHK cells were infected with 1,000, 100, 10, or 1 particle of wt HSV-1, *in*1814, or 1814R per cell, and incubation was continued for 15 h in the presence of phosphonoacetic acid to prevent the secondary spread of virus. Table 2 shows the results of TK assays performed on the cell extracts. The level of TK after infection with 1,000 or 100 particles per cell was indistinguishable for wt HSV-1, *in*1814, and 1814R, reemphasizing that *in*1814 is not detectably impaired at high MOI. At 10 particles per cell, the TK level in *in*1814-infected cells relative to that in wt HSV-1-infected cells was reduced by 6-fold, and at 1 particle per cell the decrease was 33-fold. Therefore, the expression of TK (and presumably of other early and late genes) is more strictly dependent on MOI for *in*1814 than for wt HSV-1 or 1814R, and it is likely that the observed reduction in expression is large enough to account for the inefficiency of plaque formation by the mutant.

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

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

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

Complementation of *in*1814 by Vmw110 and Vmw65. If *in*1814 fails to form plaques at low MOI because of the reduction in IE gene expression, then complementation of this state should increase the efficiency of plaque formation and consequently the apparent titer of the mutant virus. In contrast, a compensating increase in IE gene expression would not complement *in*1814 growth if the mutant phenotype resulted from a defect at a stage before the onset of IE transcription. Two experiments were carried out to test these possibilities.

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

Complementation of *in*1814 in HFL cells was achieved by infecting monolayers with UV-irradiated *ts*K, which supplied functional Vmw65 in *trans* (48), prior to titration of wt HSV-1 or *in*1814. The apparent titer of *in*1814 increased from 5.7×10^6 to 1.5×10^9 PFU/ml, whereas the titer of wt HSV-1

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

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

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

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

[a] 50% Lethal dose in terms of particles per mouse is shown in parentheses.

was 6.0×10^{10} on both cell monolayers. No plaques were observed on monolayers treated only with UV-irradiated *ts*K. The titer of *in*1814 on UV-irradiated-*ts*K-treated cells represented a particle/PFU ratio of 74, and after account is taken of the fact that the MOI for UV-irradiated *ts*K was only 0.1 PFU per cell, it is clear that the efficiency of plaque formation was similar to that of wt HSV-1. Thus, the observed phenotype of *in*1814 on HFL cells was reversed by provision of Vmw65 in *trans*, arguing against a *cis*-acting defect, for example, inhibition of uncoating by the mutant protein.

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

DISCUSSION

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

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

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

Even though *in*1814 lacks transinducing activity, the major polypeptides synthesized under IE conditions are the IE proteins. Activation by Vmw65 is therefore not a definitive characteristic of IE genes, and other features must distinguish them from early and late genes. It may be that the presence of strong promoters and enhancer-like sequences determines the relatively high efficiency of IE gene transcription in the absence of IE proteins, but equally, the TAATG ARAT or other IE-specific elements might be responsible. It is known that cellular proteins bind to various sequences in IE gene upstream regions (26, 27, 39, 49, 68), and these factors might increase the availability of IE promoters to transcription components in the absence of Vmw65. Thus, IE-specific DNA sequences, rather than Vmw65, may be the primary determinants of an IE gene.

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

levels of IE proteins. The fact that deletion mutants in Vmw110 are also impaired for growth at low MOI and show a relatively greater reduction in HFL cells than BHK cells (65; R. D. Everett, J. Gen. Virol., in press) supports the latter proposal, but further work is needed to clarify this important point.

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

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

It is interesting to speculate on the role of Vmw65 in HSV latency in the light of the phenotype of *in*1814, since the majority of genes, including IE genes, are silent during latency (62, 63), suggesting that an early transcriptional block may operate. One hypothesis is that Vmw65 may be lost or rendered inactive during transport of the HSV$_2$ nucleocapsid from the neuronal cell surface to the nucleus (27). From the analysis of *in*1814 presented here it is possible to predict that under such circumstances, at low MOI, virus replication would not ensue, and thus latency might be established. Support for this view comes from our recent observation that noninfectious particles of *in*1814 can be retained by tissue culture cells after infection at low MOI and can subsequently be reactivated to form plaques (C. Ace and C. M. Preston, unpublished results), as found in studies with a mutant lacking Vmw110 (N. D. Stow and E. C. Stow, J. Gen. Virol., in press). Therefore, the failure to transinduce IE transcription by interference with Vmw65 function is worthy of serious consideration as a basic precondition for latency.

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

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

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

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

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

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MATERIALS AND METHODS

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

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

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

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

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

Radiolabeling of viral polypeptides. For IE polypeptides, BHK cell monolayers were infected in the presence of 200 μg of CH per ml. After 4 h at 38.5°C, CH was washed from the plates and proteins were radiolabeled for 1 h with [^{35}S]methionine in the presence of 1 μg of actinomycin D per ml and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (46). For early and late polypeptides, BHK cell monolayers were infected and incubated at 37°C for 8 h, and proteins were radiolabeled for 1 h with [^{35}S]methionine and analyzed by SDS-PAGE.

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

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

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

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

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

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

RESULTS

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

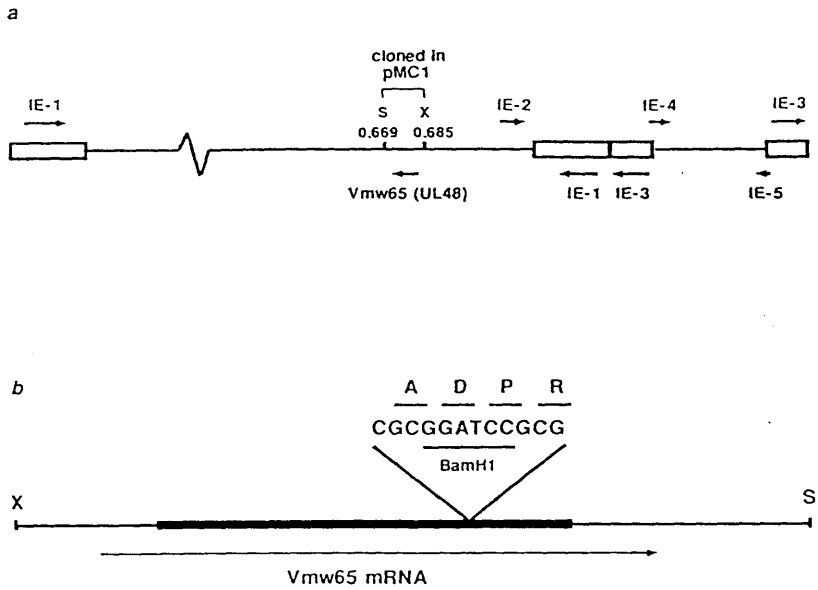

FIG. 1. (a) Structure of the HSV-1 genome showing the positions of the five IE genes (without introns) and Vmw65. The open boxes represent repeated sequences. (b) Structures of the insertion mutation in Vmw65 encoded on a *Sal*I(S)-*Xho*I(X) fragment in plasmid pMC1.in14.

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

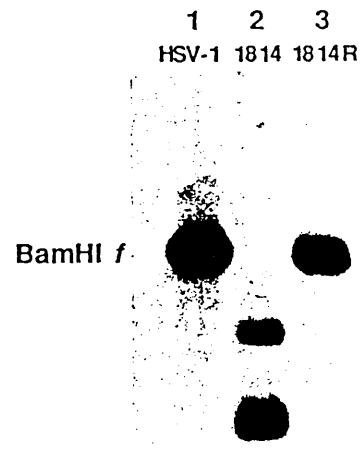

FIG. 2. Structure of the *in*1814 genome. Wt HSV-1 (lane 1), *in*1814 (lane 2), or 1814R (lane 3) DNA was cleaved with *Bam*HI, and the fragments were separated on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pMC17. The position of HSV-1 *Bam*HI *f* is indicated.

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

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

[a] ND, Not determined.

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

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

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

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

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

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

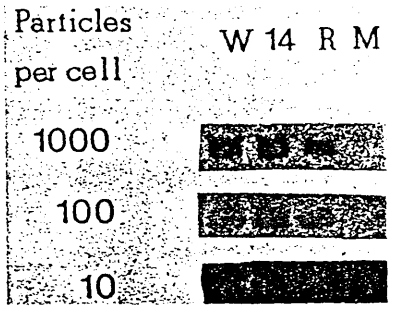

FIG. 3. DNA migration to the nucleus. DNA isolated from nuclei of cells infected with wt HSV-1 (W), *in*1814 (14), or 1814R (R) or mock infected (M), in the presence of CH, was cleaved with *Bam*HI, and the fragments were separated on an agarose gel, transferred to GeneScreen Plus membrane, and probed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

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

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

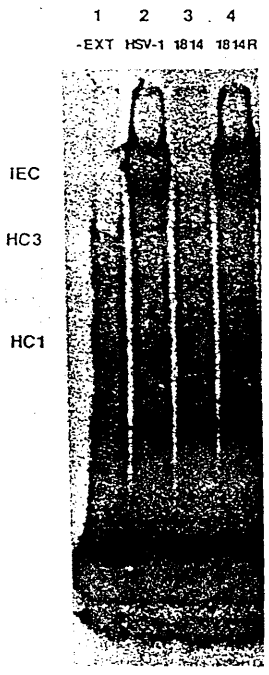

FIG. 5. IEC formation by Vmw65. A 74-bp DNA fragment containing the IE gene 4/5 TAATGAGAT sequence was incubated with HeLa cell nuclear extract alone (lane 1) or with virion extract (EXT) from wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and was analyzed by gel electrophoresis. The positions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

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

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

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

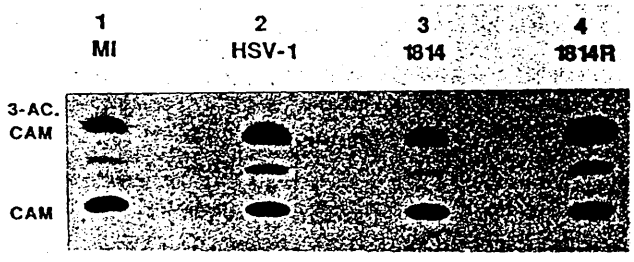

FIG. 6. Transinduction of IE transcription. Cells were transfected with pIE3CAT and mock infected (lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4). Chloramphenicol acetyltransferase assays were carried out on cytoplasmic cell extracts. The positions of chloramphenicol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

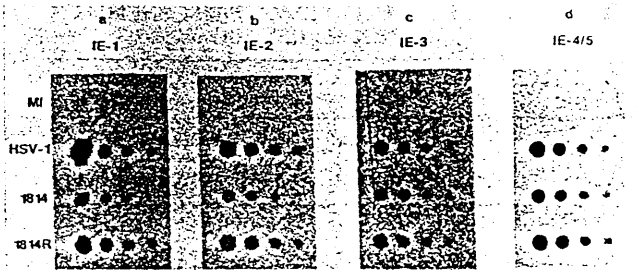

FIG. 7. Production of IE RNA. Cells were mock infected (MI; row 1) or infected with wt HSV-1 (row 2), *in*1814 (row 3), or 1814R (row 4) in the presence of CH. RNA was prepared after 4 h and applied to nitrocellulose filters in four sequential dilutions (3 μg, 1 μg, 0.3 μg, and 0.1 μg). Filters were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

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

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

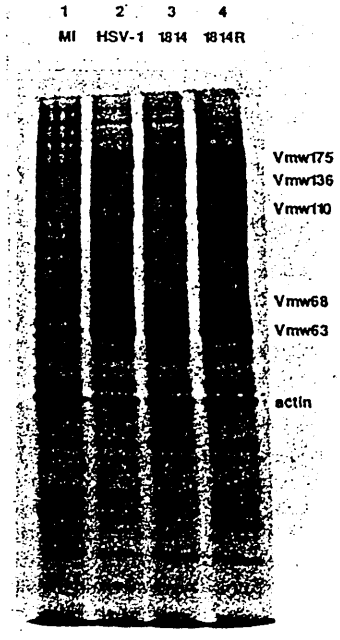

FIG. 8. IE polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4) in the presence of CH. Proteins were labeled with [^{35}S]methionine after removal of CH by washing at 4 h postinfection. The positions of viral IE polypeptides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

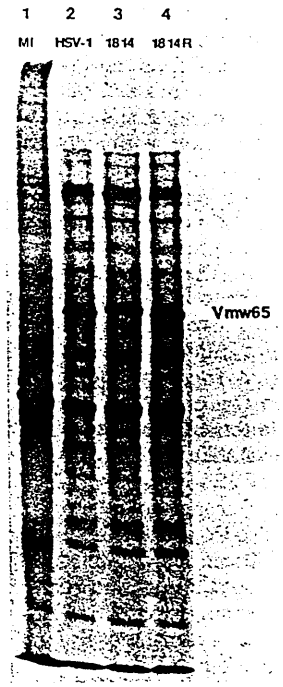

FIG. 9. Late polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and proteins were labeled with [^{35}S]methionine at 8 h postinfection. The position of Vmw65 is indicated.

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

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

Thus, upon infection of BHK cells with 1,000 particles of *in*1814 per cell in the presence of CH, the level of expression of IE genes 1, 2, 4, and presumably 5 is reduced, but under normal conditions infection procedes to the late stage, suggesting that there is no overall consequence of reduced IE gene expression. From the high particle/PFU ratio, however, it appears that the growth of *in*1814 is inefficient when cells are infected with 1 virus particle per cell. To investigate whether the incapacity of *in*1814 at low MOI is reflected in reduced gene expression, the synthesis of TK, an early enzyme that can be detected with high sensitivity, was examined. BHK cells were infected with 1,000, 100, 10, or 1 particle of wt HSV-1, *in*1814, or 1814R per cell, and incubation was continued for 15 h in the presence of phosphonoacetic acid to prevent the secondary spread of virus. Table 2 shows the results of TK assays performed on the cell extracts. The level of TK after infection with 1,000 or 100 particles per cell was indistinguishable for wt HSV-1, *in*1814, and 1814R, reemphasizing that *in*1814 is not detectably impaired at high MOI. At 10 particles per cell, the TK level in *in*1814-infected cells relative to that in wt HSV-1-infected cells was reduced by 6-fold, and at 1 particle per cell the decrease was 33-fold. Therefore, the expression of TK (and presumably of other early and late genes) is more strictly dependent on MOI for *in*1814 than for wt HSV-1 or 1814R, and it is likely that the observed reduction in expression is large enough to account for the inefficiency of plaque formation by the mutant.

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

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

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

Complementation of *in*1814 by Vmw110 and Vmw65. If *in*1814 fails to form plaques at low MOI because of the reduction in IE gene expression, then complementation of this state should increase the efficiency of plaque formation and consequently the apparent titer of the mutant virus. In contrast, a compensating increase in IE gene expression would not complement *in*1814 growth if the mutant phenotype resulted from a defect at a stage before the onset of IE transcription. Two experiments were carried out to test these possibilities.

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

Complementation of *in*1814 in HFL cells was achieved by infecting monolayers with UV-irradiated *ts*K, which supplied functional Vmw65 in *trans* (48), prior to titration of wt HSV-1 or *in*1814. The apparent titer of *in*1814 increased from 5.7×10^6 to 1.5×10^9 PFU/ml, whereas the titer of wt HSV-1

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

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

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

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

[a] 50% Lethal dose in terms of particles per mouse is shown in parentheses.

was 6.0×10^{10} on both cell monolayers. No plaques were observed on monolayers treated only with UV-irradiated *ts*K. The titer of *in*1814 on UV-irradiated-*ts*K-treated cells represented a particle/PFU ratio of 74, and after account is taken of the fact that the MOI for UV-irradiated *ts*K was only 0.1 PFU per cell, it is clear that the efficiency of plaque formation was similar to that of wt HSV-1. Thus, the observed phenotype of *in*1814 on HFL cells was reversed by provision of Vmw65 in *trans*, arguing against a *cis*-acting defect, for example, inhibition of uncoating by the mutant protein.

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

DISCUSSION

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

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

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

Even though *in*1814 lacks transinducing activity, the major polypeptides synthesized under IE conditions are the IE proteins. Activation by Vmw65 is therefore not a definitive characteristic of IE genes, and other features must distinguish them from early and late genes. It may be that the presence of strong promoters and enhancer-like sequences determines the relatively high efficiency of IE gene transcription in the absence of IE proteins, but equally, the TAATG ARAT or other IE-specific elements might be responsible. It is known that cellular proteins bind to various sequences in IE gene upstream regions (26, 27, 39, 49, 68), and these factors might increase the availability of IE promoters to transcription components in the absence of Vmw65. Thus, IE-specific DNA sequences, rather than Vmw65, may be the primary determinants of an IE gene.

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

levels of IE proteins. The fact that deletion mutants in Vmw110 are also impaired for growth at low MOI and show a relatively greater reduction in HFL cells than BHK cells (65; R. D. Everett, J. Gen. Virol., in press) supports the latter proposal, but further work is needed to clarify this important point.

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

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

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

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Control of expression of the varicella-zoster virus major immediate early gene

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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 element. 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 transciption 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; Strum *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 ATGCAAT 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 comparable their 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 pMCI 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. Reverse transcriptase

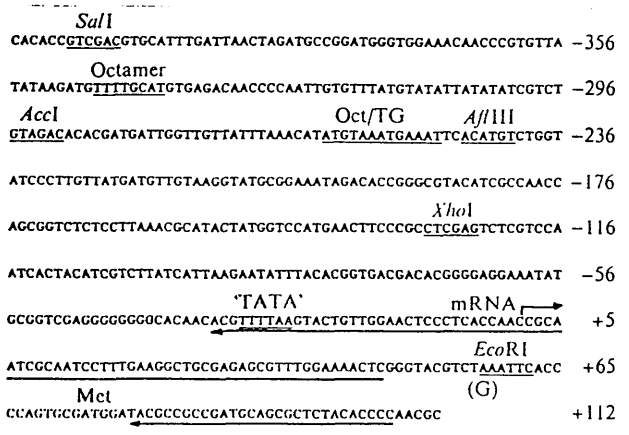

Fig. 1. DNA sequences in the upstream regulatory region of VZV ORF 62. Restriction endonuclease cleavage sites relevant to the results are underlined. The mRNA start site is designated +1 by convention and a TATA box homology TTTTAA is found at positions −25 to −30. A 13 bp sequence centred at −255 contains 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.

(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'GAGTTTTCC-AAACGCTCTCGCACCTTCAAAGGATTGCGATTGCGTTG-GTGAGGGAGTTCCAACAGTACTTAAAACGT3') 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 pMCl, 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 ATGCAAAT-CATGT (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 an 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,

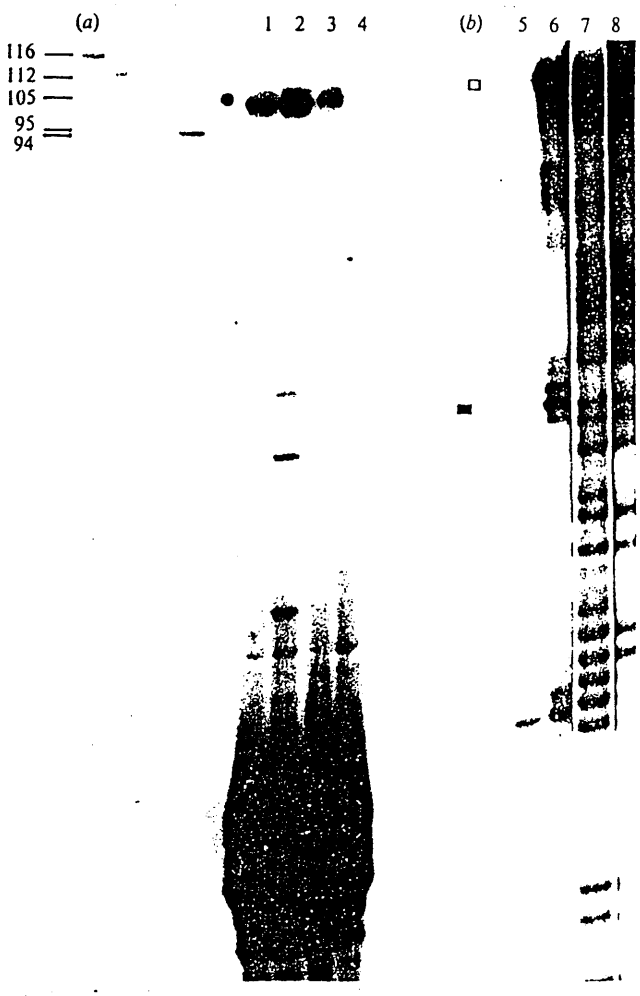

Fig. 2. Mapping the 5′ end of the mRNA. (*a*) Primer extension analysis was performed on three RNA samples prepared from different batches of VZV-infected HFL cells (lanes 1, 2 and 3) and from mock-infected HFL cells (lane 4). Markers of the indicated sizes (bp) were run in parallel and the extended 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

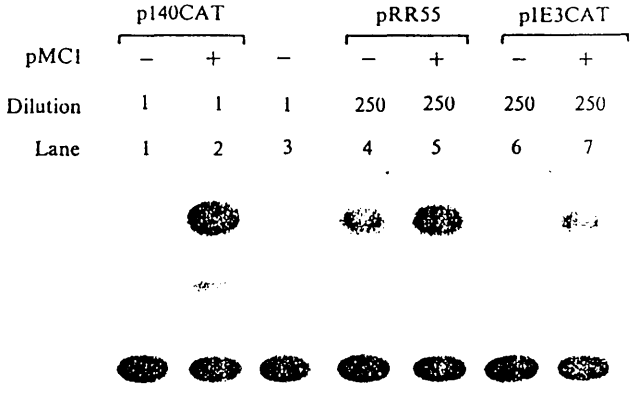

Fig. 3. Comparison of the activities of IE control sequences of VZV (lanes 1 and 2), HCMV (lanes 4 and 5) and HSV-1 (lanes 6 and 7) linked to the CAT gene. Lane 3 represents mock-transfection. In lanes marked + (lanes 2, 5 and 7) reporter plasmids were cotransfected with pMCl, whereas in lanes marked − (lanes 1, 4 and 6) pUC18 was added in order to equalize plasmid quantities.

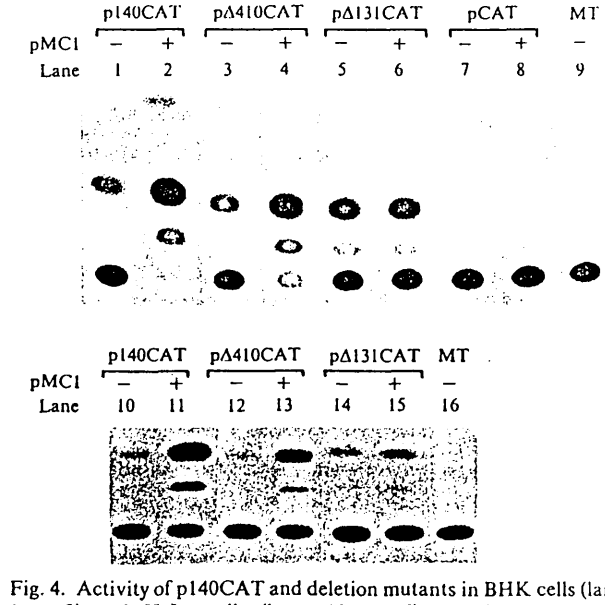

Fig. 4. Activity of p140CAT and deletion mutants in BHK cells (lanes 1 to 9) and HeLa cells (lanes 10 to 16). To demonstrate the reponsiveness of these sequences to Vmw65, cells for lanes marked + were cotransfected with pMCl and those for lanes marked − with pUC18. Samples 10 to 16 were excised from the same autoradiograph and represent equal exposures.

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, 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) and a slight extension of the region of protection occurred in the presence of Vmw65 (lane 1).

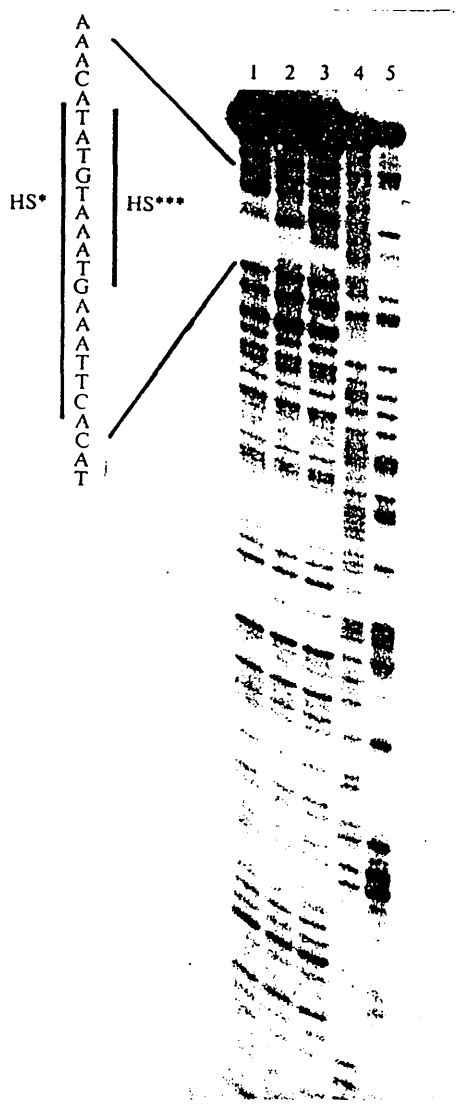

Fig. 6. DNase I footprinting analysis of a 165 bp *Acc*I/*Xho*I (−296 to −131) fragment. Lanes 4 and 5 are G and G + A sequencing tracks. Prior to DNase I treatment, the fragment in lane 1 was incubated with HeLa cell extract and virion extract, the fragment in lane 2 with HeLa extract, whereas lane track 3 represents free fragment alone. Dark bars represent the sequences protected 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 410 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

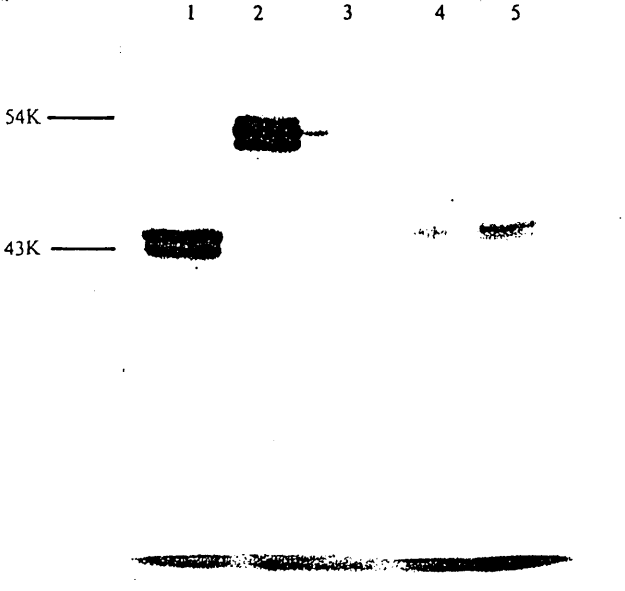

Fig. 7. *In vitro* translation of Vmw65 and ORF 65 polypeptides. RNA transcribed from pGEMTIF.in15 (lane 1), pGEMTIF (lane 2), pGEM2 (lane 3) and pGEMORF10A and -B (lanes 4 and 5) were translated *in vitro* in the presence of [^{35}S]methionine. M_r values of the proteins predicted from the DNA sequence are given. Comparison with M_r standards gives apparent M_r of 63000 (Vmw65) and 54000 (ORF 10).

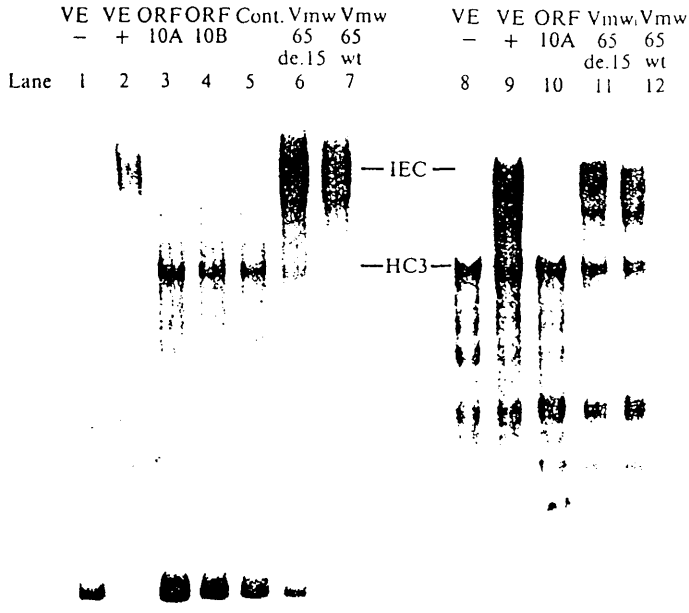

Fig. 8. Gel retardation assay using *in vitro* synthesized Vmw65 and ORF 10 products. Radiolabelled probes used on binding reactions were a 49 bp *AccI*/*Afl*III fragment from the VZV major IE gene control sequences (lanes 1 to 7) and a 74 bp fragment from HSV 1 IE control sequences (lanes 8 to 12). 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), pGEMTIF (lanes 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 (Figl 8). As expected, Vmw65 produced *in vitro* from pGEMTIF directed the formation of IEC when tested with the 49 bp *AccI*/*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, VCV 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 active 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 180 K and thus the potential for high level synthesis, which occurs when autoregulation is prevented, is not realized. In the case of VZV, ORF 62 product may accumulate more slowly, but reaches intracellular levels comparable to those of Vmw175 or 180 K. Alternatively, the low observed activity of p140CT 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). It was more suprising 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 IE transcription from 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 proteins to TAATGARAT *per se* is sufficient to confer IE specificity to a gene, perhaps by displacing repressors 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.

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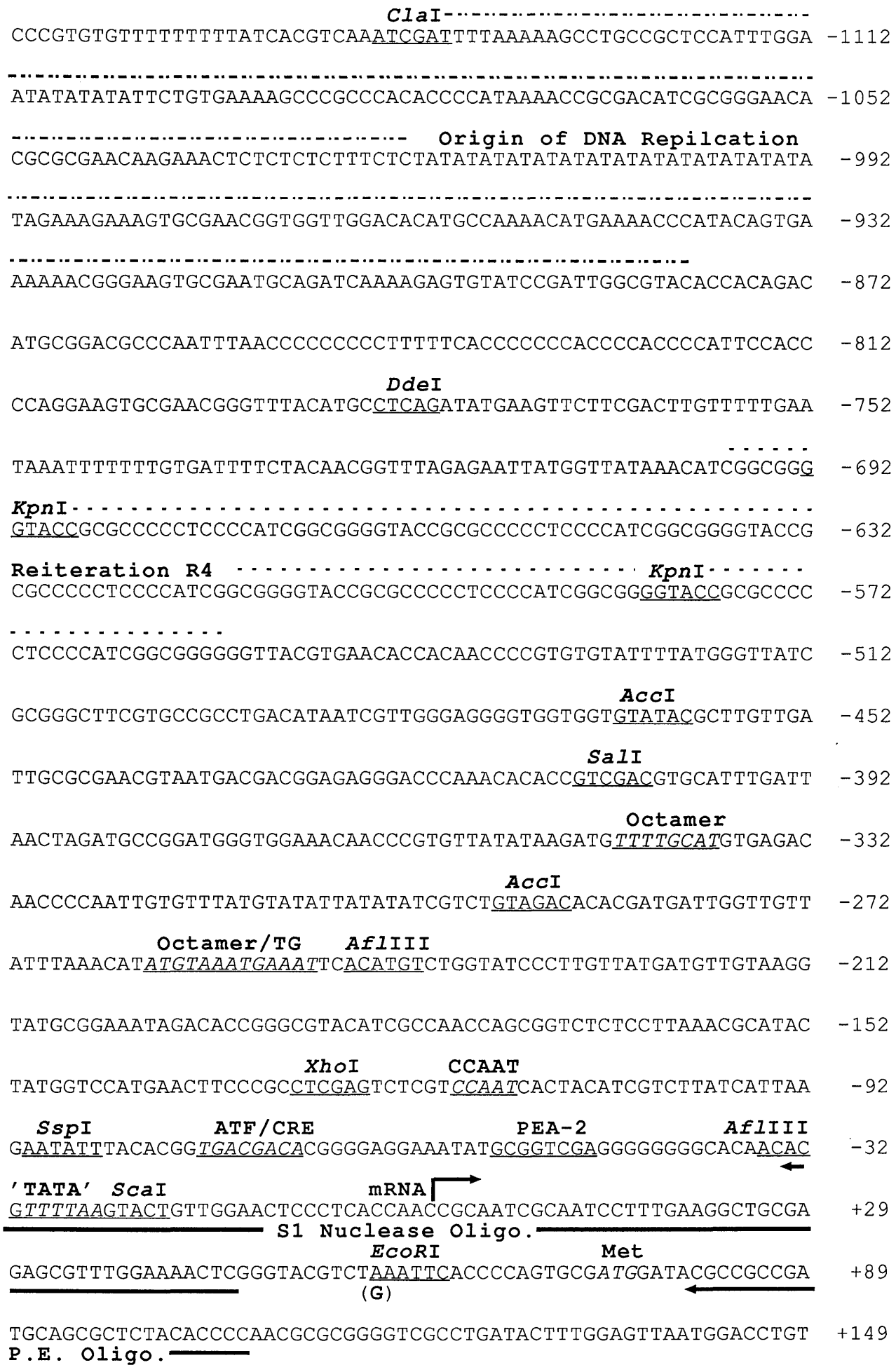
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Origin of DNA Repilcation
CGCGCGAACAAGAAACTCTCTCTCTTTCTCTATATATATATATATATATATATATATA -992
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DdeI
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KpnI
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Reiteration R4
KpnI
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AccI
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SalI
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Octamer
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AccI
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Octamer/TG
AflIII
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XhoI
CCAAT
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SspI
ATF/CRE
PEA-2
AflIII
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'TATA'
ScaI
mRNA
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S1 Nuclease Oligo.
EcoRI
Met
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(G)
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P.E. Oligo.