A MUTATIONAL ANALYSIS OF THE STRUCTURE AND FUNCTION OF THE HERPES SIMPLEX VIRUS IMMEDIATE EARLY PROTEIN Vmw175

by

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SUMMARY

Herpes simplex virus type 1 (HSV-1) expresses three main classes of genes (immediate-early [IE], early [E] and late [L]) in a temporal cascade upon infection of tissue culture cells. These three groups of genes can also be defined by the sensitivity of their expression to metabolic inhibitors of protein or DNA synthesis. IE genes are transcribed in the absence of de novo protein synthesis and their transcription is stimulated by a component of the virus particle, Vmw65. IE gene products are required for the activation of later classes of viral genes, whose expression varies in sensitivity to inhibitors of viral DNA replication. Early genes are expressed at maximal levels in the absence of DNA synthesis, whilst true late genes are critically dependent on replication for expression.

The predominant transcriptional regulatory protein specified by HSV-1 is the IE protein Vmw175, whose functions are essential for virus growth. Studies of viruses with temperature sensitive lesions in this protein have shown that Vmw175 is a complex multifunctional protein required for the transcriptional activation of many HSV-1 promoters and the repression of its own transcription. Cloned Vmw175 is a promiscuous transactivator of transcription of RNA polymerase type II promoters. In addition cloned Vmw175 represses transcription from its own promoter, probably by binding to a specific target sequence at the start site for transcription.

The aim of the work described in this thesis was to investigate the relationship between the structure and function of this protein, and to define which regions of the protein are involved in each of its various activities. A panel of plasmid-borne in-frame insertion and deletion mutants of the gene encoding Vmw175 were constructed and assayed for their ability to regulate transcription in transient transfection assays. Fusions of HSV promoters to the chloramphenicol acetyl transferase gene were used to assay the ability of each mutant to transactivate an HSV early promoter (that of the gene encoding glycoprotein gD) and to repress the promoter of its own gene, IE3, in transiently transfected cells. By this approach it was

possible to define the regions of the Vmw175 amino acid sequence that are required for transcriptional activation and repression. Large stretches of the protein are relatively unimportant for either function, while the regions most sensitive to disruption correlate to sequences conserved between Vmw175 and VZV 140K, the corresponding transactivating protein of another alphaherpesvirus, varicella-zoster virus. The region from amino acids 275 to 495 is particularly important for both repression and transactivation; whilst that from around 840 to 1100 seems to be more important for transactivation than repression.

A monoclonal antibody directed against Vmw175 was used to visualize expression and localization of Vmw175 in transfected cells by indirect immunofluorescence. This provided confirmation that each of the mutant plasmids expressed Vmw175. Each of the 39 insertion mutant proteins exhibited a pattern of nuclear localization indistinguishable from that of wild-type, but when amino acids 682-774 were deleted a signal essential for nuclear localization was lost. A strong candidate for a nuclear localization signal centres around amino acid 728 and is strongly conserved in the VZV homologue. This sequence PREGRKRKSP contains four consecutive arginine (R) and lysine (K) residues and is related to a signal in the SV40 large T antigen required for nuclear localization.

Vmw175 is known to bind directly to a number of HSV-1 sequences, some of which contain the consensus sequence ATCGTC. The binding of Vmw175 to this sequence at the transcriptional start site of IE gene 3 is thought to be involved in the mechanism of autoregulation. In order to correlate the transcriptional activity of each mutant with its ability to bind to DNA, the site specific DNA binding activity of each mutant was assayed. In these experiments nuclear extracts of transfected cells were incubated with a DNA probe spanning the IE3 cap site and protein complexes detected using the gel retardation technique. The results show that a critical region of Vmw175, amino acid residues 275-495, includes structures which are essential for specific DNA binding, transactivation and repression. This raises the interesting paradox that although the specific DNA sequence recognized by Vmw175 is not commonly found

in its target promoters, the protein domain required for recognition of this sequence is required for promoter activation.

Work was initiated to recombine some of these plasmid-borne mutants back into the viral genome. To facilitate this an HSV-1 deletion mutant lacking both copies of the gene was constructed and the plasmid-borne mutations were transfered into this virus by homologous recombination in transfected cells. The long term aim of constructing these viruses was to use them as vectors to produce large amounts of mutant Vmw175. Three novel recombinant viruses were constructed in this manner and their characterization is described. The most interesting virus contained a 68 codon deletion in both copies of the gene encoding Vmw175. Although this deletion removed a conserved serine-rich phosphorylation site in Vmw175 this virus was viable when grown on a non-complementing cell-line, confirming the conclusion drawn from study of this mutation in transient transfection assays that this region of the protein is functionally non-essential.

The implication of these results regarding the structure of Vmw175 and the possible mechanisms by which this protein might function to regulate transcription are discussed.

ABBREVIATIONS

A	adenine
ABTS	2,2'-azinobis(3-ethylbenzthiazocine sulphonic acid)
Ac	acetate
Ad	adenovirus
Ap	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BAP	bacterial alkaline phosphatase
BES	N,N-bis(2-hydroxyethyl)2-aminoethanesulphonic acid
BHK	baby hamster kidney cells
bp	base pairs
BSA	bovine serum albumin
C	cytosine
^{14}C	Carbon-14 radioisotope
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
C_t	carboxy-terminal (of a polypeptide)
cav	cell associated virus
CAT	chloramphenicol acetyl transferase
CBP	CCAAT binding protein
CHX	cycloheximide
Ci	Curie (s)
Cm	chloramphenicol
CMV	cytomegalovirus
cpe	cytopathic effect
cpm	counts per minute
crv	cell released virus
CS	calf serum
CTP	cytidine-5'-triphosphate
Da	Daltons
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
E	early (gene)

EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	sodium ethylenediamine tetra-acetic acid
EL	early-late (gene)
ELISA	enzyme linked immuno-sorbent assay
ER	oestrogen receptor
ERE	oestrogen response element
EtBr	ethidium bromide
FCS	foetal calf serum
G	guanine
g	grams
GlcNAc	N-acetylglucosamine
GMEM	Glasgow modification of Eagle's medium
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h,hr	hour(s)
HAT	hypoxanthine, aminopterin, thymidine (selection)
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRE	hormone response element
HSV	herpes simplex virus
HZ	hydrazine
I	inosine
IE	immediate early (gene)
IEC	immediate early complex (on TAATGARAT elements)
Ig	immunoglobulin
IR	internal repeat
k	kilo
kb	kilobase
l	litre
L	late (gene)
LFP	large (Klenow) fragment of DNA polymerase
LTR	long terminal repeat
M	molar
MDBP	major DNA binding protein
min	minute(s)
ml	millilitre
ML	major late (adenovirus transcription unit)
mm	millimetre
mM	millimolar
moi	multiplicity of infection

mol	moles
MOPS	3-(N-morpholine)propanesulphonic acid
mRNA	messenger ribonucleic acid
MRE	metal response element
M.Wt.	molecular weight
N	unspecified nucleotide or amino acid
n	nano
NBC	new born calf serum
NE	nuclear extract
ng	nanogram
NPT	non-permissive temperature
NP40	Nonidet p40
nt	nucleotide
N_t	amino-terminal (of a polypeptide)
OD	optical density
ORF	open reading frame
ORI	origin of replication
^{32}P	Phosphorus-32 radioisotope
p.a.	post-absorption
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
pmol	picomole
PMSF	phenylmethylsulphonyl fluoride
poly(A)	polyadenylic acid
ppt	precipitate
PrV	pseudorabies virus
PT	permissive temperature
R	purine moiety
RE	restriction enzyme
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNAase	ribonuclease
rpm	revolutions per minute
RR	riboncleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
sec	seconds
syn	syncytial plaque morphology locus (syn^+ = non-syncytial, syn^- = syncytial)

SV40	simian virus 40
T	thymine
TEMED	n,n,n',n'-tetramethylethylene diamine
tk	thymidine kinase
TLC	thin layer chromatography
TPA	12-O-tetradecanoyl-phorbol-13-acetate
T_3R	Thyroid hormone receptor
Tris	tris(hydroxymethy)aminomethane
tRNA	transfer ribonucleic acid
ts	temperature sensitive
UAS	upstream activating sequence
UPE	upstream promoter element
uv	ultraviolet
V	volt
v	volume
VIC	virally induced complex (on TAATGARAT elements)
Vmw	apparent molecular weight of virus-induced protein
vol	volume
VP5	virion protein 5 (major capsid protein)
VZV	varicella zoster virus
w	weight
W	watts
wt/WT	wild type
Y	pyrimidine moiety
u	micro

AMINO ACID SYMBOLS

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

(The three letter code abbreviations are underligned).

CHAPTER 1: INTRODUCTION

The research presented in this thesis concerns the role and function of the predominant transcriptional regulatory protein of HSV-1, the immediate-early protein Vmw175. As such it is necessary to consider the role of Vmw175 in relationship to the viral life-cycle and also in relationship to the host-cell transcriptional machinery. This introduction, therefore, aims to describe briefly the biology of HSV-1, with particular reference to its gene regulation and transcriptional programme, and also to review the current knowledge of the eukaryotic transcriptional machinery and possible mechanisms by which transcriptional regulation may function.

SECTION 1A: THE BIOLOGY OF HSV-1

1. Classification and structure of the herpesviruses

Members of the Herpesviridae form a morphologically distinct virus family characterized by the inclusion of a linear, double-stranded DNA genome in an enveloped virion particle 150-200nm in diameter. The virus particle is composed of four distinct elements: an electron dense core containing the genomic DNA (Epstein, 1962) an icosadeltahedral capsid comprizing of 162 capsomeres (Wildy et al., 1960) an undefined proteinaceous tegument surrounding the capsid (Roizman and Furlong, 1974) enveloped in a trilaminar membrane deriving from the host-cell's nuclear membrane (Wildy et al., 1960) but containing numerous virally encoded glycoprotein spikes (Spear and Roizman, 1972).

The herpesviruses comprize a large family with more than eighty identified members exhibiting diverse hosts and biological properties. Never-the-less, in addition to conserving a distinct morphological structure and intranuclear replicative cycle the herpesviruses are distinguished by sharing the ability to persist in a latent state in the infected host.

The herpesviruses have historically been subdivided on the basis of their biological properties into three

subfamilies (Mathews, 1982; Roizman, 1982). HSV-1 and 2 are members of the alphaherpesviruses, characterized by a short reproductive cycle, typically less than 24h in cell culture, and latency is frequently established in ganglia. Varicella-zoster virus, the human virus responsible for chickenpox and shingles is also an alphaherpesvirus.

The betaherpesviruses have a restricted host range and a longer reproductive cycle, with infection spreading slowly in culture. Latency can be established in a variety of tissues. The cytomegaloviruses belong to this subfamily.

Gammaherpesviruses have a narrow host range, generally restricted to B or T lymphocytes, and latency is frequently established in lymphoid tissue. Epstein-Barr virus is the human prototype for this subfamily and causes infectious mononucleosis.

More recently the evolutionary relationship between members of the subfamilies has been demonstrated at the level of genome organization and DNA sequence. Classification on these bases and the serological cross-reactivity of virus gene products is of increasing importance (Roizman, 1982). Indeed it is now apparent that classification on the basis of biological properties alone may not truely reflect phylogenetic relationships. For example selective sequence analysis of the recently discovered human lymphotropic virus, HHV-6 (Salahuddin et al., 1986) has revealed that it is probably related to the betaherpesvirus lineage (Honess et al., XIIIth International Herpesvirus Conference) although it does share some reiterated sequences with Marek's Disease Virus (Kishi et al., 1988) and in its tissue tropism more closely resembles the gammaherpesviruses.

2. HSV-1 Genome structure and organization

The herpesvirus genome can be extracted from virus particles as a single, linear, double-stranded DNA molecule, which does, however, contain single-stranded nicks and gaps (Kieff et al., 1971; Wilkie, 1973). The structure of these molecules has been of considerable interest for many years, since hybridization studies and electron microscopy revealed extensive sequence reiterations or repeats in the

herpesvirus genomes (Sheldrick and Berthelot, 1974).

The sequence arrangements of the herpesviruses can be described by one of a number of models, depending on the extent and form of the sequence reiterations and the ability of the genome to isomerize (for a full description see Roizman and Batterson, 1985). The original model for the HSV genome structure shown in Figure 1 (Sheldrick and Berthelot, 1974) has been proved substantially correct by later studies (Wadsworth et al., 1975; Wilkie, 1976).

The genome can be considered as consisting of two covalently linked segments L (long) and S (short) which can invert relative to each other to give four different genome isomers. The unique sequences of the long and short segments (U_L and U_S) are flanked by distinct inverted repeats R_L and R_S. Each end of the genome is flanked by a direct repeat sequence of variable length (Davison and Wilkie, 1981). Whilst a single copy of this a sequence is found at the S terminus, multiple copies are detected at the L terminus, and a variable number in inverted orientation at the LS junction. The a sequence itself is composed of a complex pattern of tandemly reiterated sequences which seem to account for an overall variability of genome length.

Representative isolates of four herpesviruses have now been completely sequenced: EBV (Baer et al., 1984) VZV (Davison and Scott, 1986) HSV-1 (McGeoch et al., 1988b) and human CMV (Barrell et al., per.comm.). The HSV-1 strain 17 syn^+ was found to have a prototype length of 152 260 bp, which will vary dependent upon the sequence reiterations, and it has a G+C content of 68.3%. The coding potential of the genome has been analysed in detail making use of the sequence data and earlier mapping data (McGeoch et al., 1985, 1988b). It has been proposed that there are 72 protein-coding genes in the HSV-1 genome, comprizing 70 unique genes of which two are represented twice (IE110 and IE175) (Figure 2). These open reading frames determined by sequence analysis have been assigned the names UL1-56, IE110, IE175 and US 1-12, but many of these "genes" have not yet been shown to direct polypeptide synthesis, nor assigned a function.

Study of the genome map in Figure 2 shows that HSV-1

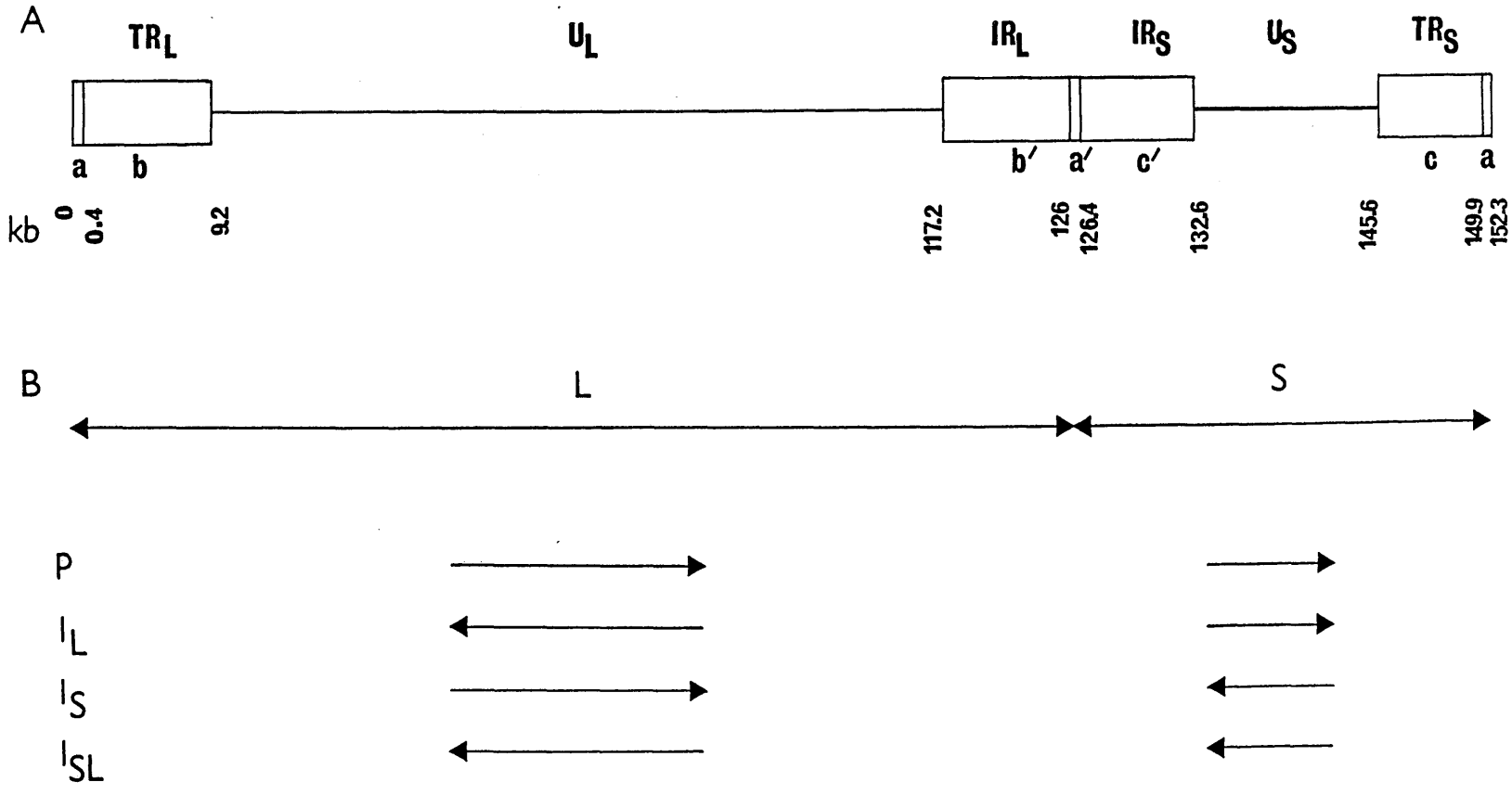


Figure 1: Genome structure of HSV. (A) The genome arrangement of HSV-1. The long region (L) is composed of the unique region (U_L) bounded by a terminal sequence (TR_L) which is repeated internally in an inverted orientation (IR_L). Similarly the short region (S) is composed of a unique region (U_S) which is bounded by a terminal sequence (TR_S) which is internally repeated in an inverse orientation (IR_S). The terminally redundant sequences are designated a/a', and the remaining sequences within TR_L/IR_L and TR/IR_S are designated b/b' and c/c' respectively. (B) The four genome isomers are shown which can be formed by inversion of the L and/or S segments. (P: prototype; I_L: inversion of L; I_S: inversion of S; I_{SL}: inversion of both S and L segments.)

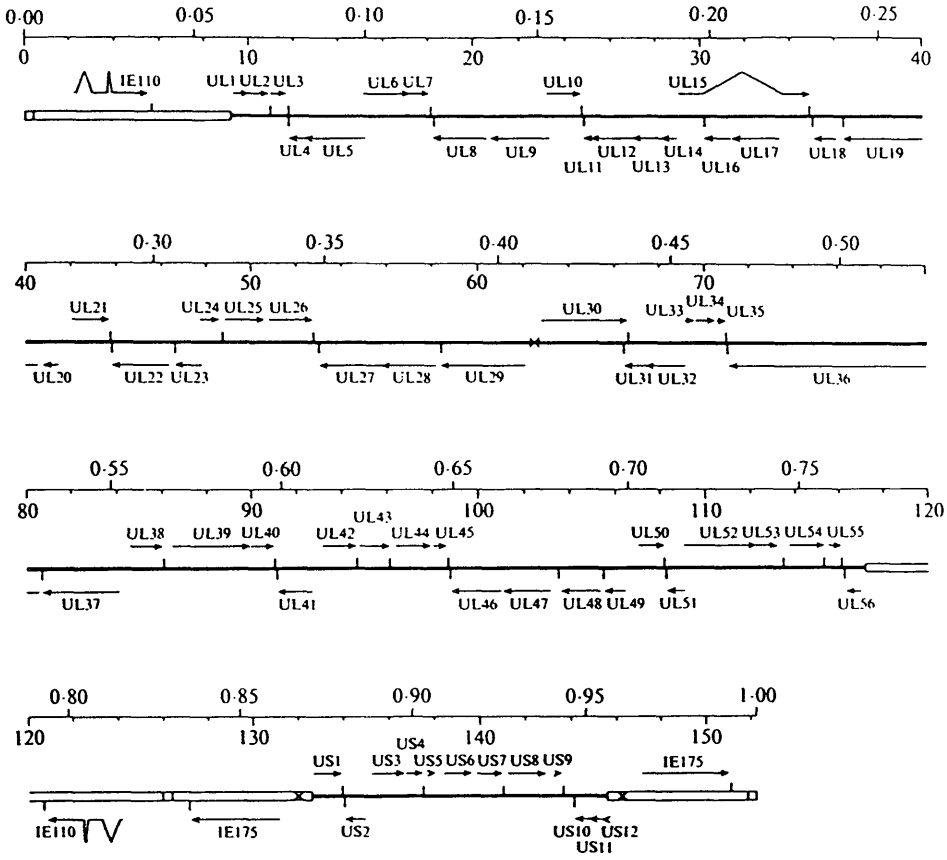


Fig. 2. Layout of genes in the genome of HSV-1. The HSV-1 genome is shown on four successive lines, with unique regions represented by solid lines and major repeat elements as open boxes as in Fig. 1. The lower scale represents kilobases, numbered from the left terminus, and the upper scale represents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

efficiently utilizes its coding capacity and few regions of the genome are noncoding. However, relatively few open reading frames overlap so genome size does not appear to be an overriding evolutionary constraint. Indeed several HSV encoded genes have been shown to be dispensible for growth, at least in actively dividing tissue culture cells (reviewed by McGeoch et al., 1988b). In particular the majority of U_S seems to encode inessential genes (Longnecker and Roizman, 1986; 1987; Weber et al., 1987).

It is noteworthy that comparison of the VZV and HSV-1 DNA sequences reveals extensive conservation between these two alphaherpesviruses both in genome organization and in the sets of genes encoded, although the actual DNA sequences have diverged to the extent that VZV DNA is composed of 46.0% G+C residues whilst HSV-1 is 68.3% G+C (Davison and Scott, 1986; McGeoch et al., 1988b). The DNA sequence of the gammaherpesvirus, EBV, however, shows considerable divergence from both of the alphaherpesviruses, both in the sets of genes present and in their genome arrangement (Davison and Taylor, 1987; McGeoch et al., 1988b).

The HSV-1 and HSV-2 genomes are highly conserved, showing at least 40-50% sequence homology as estimated by hybridization in solution (Kieff et al., 1972). Furthermore, the genomes appear to be essentially colinear from the sequence data available and homology studies (Davison and Wilkie, 1983b). The DNA sequences of the U_S regions of HSV-1 and HSV-2 have been compared in detail (McGeoch et al., 1985, 1987) and found to be colinear and conserved at the sequence level, especially within the protein coding sequences. The colinearity and homology of the HSV-1 and HSV-2 genomes is demonstrated by the ability to readily recover intertypic recombinants between the two strains (Timbury and Subak-Sharpe, 1973; Halliburton et al., 1977; Morse et al., 1977).

3. Pathogenicity

Six herpesviruses are known which infect man, including representatives from each of the three subfamilies: HSV-1, HSV-2 and VZV (alpha); CMV and probably HHV6 [HBLV] (beta); and EBV (gamma).

The close genetic relationship between HSV-1 and HSV-2 is reflected in some overlap of their clinical manifestations (reviewed by Whitley, 1985). HSV-1 typically causes vesicular lesions of the lips and mouth, known as "cold sores", whilst HSV-2 is associated with genital disease. Latency is commonly established in the neural ganglia following primary infection, with reactivation periodically occuring to give rise to recurrent lesions at the primary site of infection or other peripheral sites. Man is the sole reservoir for the transmission of HSV, which occurs during close physical contact. Infection is generally mild and rarely fatal, except in immunosuppressed individuals. HSV-1 is widespread in the human population and it has been estimated that one third of the world's populations have recurrent infections.

The other human alphaherpesvirus, VZV, manifests as two main diseases, chickenpox (varicella) and shingles (herpes zoster). Varicella is the result of primary infection typically in childhood, whilst reactivation of latent VZV is the apparent cause of herpes zoster (reviewed by Gelb, 1985).

4. Latency

In common with other herpesviruses HSV can establish and maintain a latent infection where the viral genome remains associated with the host without causing symptoms until reactivation occurs and the lytic cycle is initiated (reviewed by Hill, 1985). The viral genome is maintained in the cells of the neuronal ganglia in a non-infectious state (Stevens and Cook, 1971; Baringer and Swoveland, 1973; Cook et al., 1974). Since the terminal fragments of the HSV genome can not be detected in latent cells it is apparent that the DNA is maintained in an "endless" rather than linear form (Rock and Fraser, 1983; 1985; Efstathiou et al., 1986). It is thought that this does not reflect the integration of the HSV genome into cellular DNA but might indicate that the molecule is maintained in an extrachromosomal form, as either a circle or concatemer of the linear genome (Mellerick and Fraser, 1987).

Expression from the latent virus is severely restricted.

Recently, however, latency associated transcripts (LAT) have been detected in the nuclei of cells from latently infected mouse, rabbit and human ganglia (Stevens et al.,1987; Rock et al., 1987; Spivack and Fraser, 1987; Steiner et al., 1988). These transcripts derive from a region downstream from IE110 in R_L, an area of the genome of previously unassigned function, and are partially complementary to and overlap with the IE110 transcript. Although open reading frames do exist in this region, there is little evidence for a convincing protein coding sequence (McGeoch et al., 1988b; Weschler et al., 1988) and unique polyadenylated LAT messages cannot be detected in latently infected cells (Wagner et al., 1988).

Interest is currently focussed on the role that these two genes, LAT and IE110, may have in latency, and indeed whether they might interact to control establishment or reactivation of latency. However, it has recently been shown that a virus mutant unable to express LAT is able to cause latent infection in mice and be reactivated from explanted ganglia (Javier et al., 1988). Therefore it would appear that LAT is not an absolute requirement for establishment of the latent state.

The functions of HSV latency (establishment, maintenance and reactivation) have been studied at an organismal level using animal models and also at a cellular level using in vitro models where productive viral replication is repressed by drugs or elevated temperatures (Wigdahl et al., 1982; Shiraki and Rapp, 1986; Russell and Preston, 1986). These models allow the characterization of functions required or dispensible for latency. So far it has not been possible to identify any gene products uniquely required for establishment of latency (Polvino-Bodnar et al., 1987; Meignier et al., 1988; Russell et al., 1987b) but Vmw110 has been implicated in the reactivation of HSV in vitro (Russell et al., 1987b).

5. Transformation and oncogenesis

The herpes simplex viruses, particularly HSV-2, have for a long time been associated with human cancers, especially squamous cell carcinoma of the cervix (reviewed Rawls, 1985;

Macnab, 1987). Evidence supporting a direct role for HSV-2 in the development of cervical carcinoma is hard to evaluate; although there is a good correlation between past infection with HSV-2 and later carcinoma development, the risk factors involved in contracting a genital HSV-2 infection seem to be similar to those for developing cervical carcinoma. The expression of HSV gene products and indeed the presence of HSV DNA is only detectable in a low proportion of cervical carcinoma tissues, whereas human papilloma virus sequences are commonly detected and implicated to greater extent than HSV in carcinogenesis (reviewed by Macnab, 1987).

HSV does not seem to encode a transforming oncogene analogous to those transduced by retroviruses or encoded by DNA tumour viruses such as the papova or adenoviruses. HSV is clearly able to transform cultured cells and one region of the HSV-1 genome (MTR-I) and two regions of the HSV-2 genome (MTRs II and III) have been defined which can induce transformation. MTR-I spans map units 0.31-0.42, encompassing several genes (see Figure 2) (Reyes et al., 1979; Galloway and McDougall, 1983). Various subfragments of this region have been investigated and been shown to be mutagenic and to reactivate endogenous viruses (reviewed Macnab, 1987). The HSV-2 MTRs II and III span restriction fragments BglII n and c, in a region of the genome which encodes for both ribonucleotide reductase subunits. However, no requirement for the continuing expression of either subunit has been demonstrated. One favoured model has been that potential stem loop structures encoded in this region could function as a mutagen or as a transcriptional activator (Galloway et al., 1984). However, there is no evidence that these potential secondary structures have any significance and indeed it is possible to derive similar structures for many random DNA sequences (Macnab, 1987).

The relevance of the in vitro transformation experiments to the in vivo carcinogenesis activity of HSV has not been adequately demonstrated. Suprizingly small fragments of DNA, unable to encode any discreet protein, are able to induce transformation of cultured cells and the retention of HSV DNA is not necessary for maintenance of the transformed

state (Cameron et al., 1985). Similarly no set of genes is consistently retained or expressed in the transformed cells of human cervical tumours (reviewed by Galloway and McDougall, 1983). This has led to the proposition that HSV tranforms cells by a "hit and run" mechanism whereby a temporary association of HSV may be sufficient to transiently or permanently disrupt normal cellular gene regulation, providing the opportunity for other events to occur which might cooperate to produce an oncogenically transformed cell (Skinner, 1976; Galloway and McDougall, 1983).

There are many possible mechanisms by which HSV might disrupt normal cellular gene regulation. Several HSV gene products clearly have the potential to be mutagenic, being involved in DNA metabolism or rearrangment. It is possible that such activities could disrupt the regulation of normal gene expression in infected cells, or perhaps even cause gene rearrangments or amplifications. HSV has been shown to act as a mutagen on a cellular reporter gene (Pilon et al., 1985; 1986) and also to induce chromosome damage (Peat and Stanley, 1986).

HSV also has the potential to elevate the expression of various cellular genes upon infection (LaThangue et al., 1984; Kennedy et al., 1985; Macnab et al., 1985; Patel et al., 1986; Latchman et al., 1987). This could occur either as a function of the HSV encoded transcriptional regulators (Latchman et al., 1987) or as a consequence of mutagenic activity (discussed above). Indeed HSV-transformed cell lines have been shown to transcribe certain cellular genes at elevated levels (Filion et al., 1988) and some of the cellular polypeptides expressed at increased levels in infected cells also accumulate to higher levels in transformed cells (Macnab et al., 1985).

SECTION 1B: LYTIC INFECTION BY HSV-1

1. Virus adsorption, penetration and uncoating.

The adsorption of virus particles onto the cell surface and the subsequent penetration of the virus through the cell membrane are separable functions of the virally encoded glycoproteins present on the virus envelope. Virus adsorption to the cell surface is a rapid process, and attachment may initially be fairly non-specific, leading to weak then irreversible binding of the virion to the cell membrane (Hochberg and Becker, 1968; Rosenthal et al., 1984).

The cellular receptors for HSV attachment are not yet known but appear to be different for HSV types 1 and 2 on the basis of heterotypic competition experiments (Vahlne et al., 1979; Addison et al., 1984). Neomycin blocks the receptor binding of type 1 but not type 2 HSV, implying that phosphoinositides have a role in the type 1 virus receptor interaction (Langeland et al., 1987).

Penetration of the virus into the cell cytoplasm appears to occur by membrane fusion rather than phagocytosis, with virus envelope glycoproteins being transfered to the cell membrane (Para et al., 1980). Glycoprotein gD is essential for virus penetration, but not attachment (Ligas and Johnson, 1988; Johnson and Ligas, 1988), and neutralizing monoclonal antibodies against gD block virus entry but not cell surface attachment (Highlander et al., 1987). Cell lines expressing gD are also refractory to virus entry by fusion, with the attached particles being endocytosed and degraded (Campadelli-Fiume et al., 1988a). Glycoprotein D constitutively expressed in these cell-lines is sufficient to induce spontaneous fusion of cells to form polykaryocytes (Campadelli-Fiume et al., 1988b). The glycoprotein gB has also been implicated in virus fusion (Johnson et al., 1984; Cai et al., 1988) and syncitial plaque morphology mutations have been mapped to the gene encoding gB (DeLuca et al., 1982; Bond et al., 1982; Bzik et al., 1984) although no syncitial plaque morphology mutants have been isolated in the gene encoding gD.

After penetration the virus capsids are translocated to

the nucleus where the viral DNA is uncoated and extruded through the nuclear pore into the nucleus (Knipe et al., 1981; Batterson et al., 1983).

2. Effects of HSV infection on host cell macromolecular synthesis

Upon lytic infection of permissive cells HSV subverts the normal host cell metabolism to facilitate efficient replication of the virus genome. Host macromolecular synthesis declines, typically within 2-4 hours post infection, dependent upon virus strain and cell type (reviewed by Fenwick, 1984).

Cellular DNA synthesis is inhibited (Roizman and Roane, 1964) and indeed HSV infection induces specific chromosome damage at early times of infection, and more general and severe damage at later times (Peat and Stanley, 1986). Host cell mitosis is arrested (Wildy et al., 1961).

A general decline in host mRNA accumulation is observed upon HSV infection (Pizer and Beard, 1976; Fenwick, 1984). However, the transcriptional activation of several cellular genes during infection has been reported. In some cases this activation is dependent upon expression of the viral immediate early transactivating protein Vmw175 (Latchman et al., 1987; Kemp and Latchman, 1988) and in some cases activation is independent of viral protein synthesis (Kemp et al., 1986). Of particular interest is the induction of cellular stress and heat shock proteins by HSV infection (Notarianni and Preston, 1982; LaThangue et al., 1984; Patel et al., 1986; Kennedy et al., 1986; Russell et al., 1987a) and cellular proteins which seem to be overexpressed in HSV transformed cells (Macnab et al., 1985). It has also been shown that cellular promoters reintroduced into the genome of biochemically transformed cell-lines can be activated by HSV infection, and that this activation involves the function of the IE transactivating proteins Vmw110 and Vmw175 (Everett, 1985).

A phenomenon of major interest is the efficient shut-off of the majority of host-specified polypeptide synthesis upon infection of cells with HSV (Sydiskis and Roizman, 1966, 1967) which is accompanied by degradation of cytoplasmic

mRNAs (Nishioka and Silverstein, 1977, 1978; Schek and Bachenheimer, 1985) and disaggregation of host polyribosomes (Sydiskis and Roizman, 1967). Mitochondrial RNA species are also degraded by a post transcriptional mechanism (Latchman, 1988). The degradation of cellular mRNAs is not specific to host transcripts, and the resultant rapid turnover of viral transcripts may be important for responsive viral gene regulation (see below).

There are at least two components involved in the shut-off of host polypeptide synthesis by HSV: an "early" shut-off function involving a virion associated factor which causes a rapid degradation of host polyribosomes and reduction in host mRNA levels, and a delayed "late" shut-off function which requires expression of early and late viral polypeptides (Nishioka and Silverstein, 1978; Read and Frenkel, 1983). The mechanisms of host shut-off may differ in detail between HSV type 1 and 2 (Hill et al., 1983, 1985) and some strains of HSV-2 seem to induce a more rapid and severe inhibition of host protein synthesis (Pereira et al., 1977; Schek and Bachenheimer, 1985). HSV-2 strain G seems to encode a particularly effective virion associated host shut-off (vhs) function, which also destabilizes viral IE mRNAs in the absence of de novo viral protein synthesis (Fenwick and Owen, 1988).

Mutants in a gene responsible for virion associated shut-off (vhs) have been identified (Read and Frenkel, 1983) but are not deficient in the delayed shut-off activity. The vhs factor appears to be responsible for both non-specific cellular and viral mRNA degradation and polyribosome dissagregation (Schek and Bachenheimer, 1985; Strom and Frenkel, 1987). Viral as well as host mRNAs are more stable in vhs mutants, and the failure of these mutants to shut-off IE polypeptide expression indicates the importance of the shut-off for the rapid modulation of viral gene expression (Kwong and Frenkel, 1987; Oroskar and Read, 1987).

The vhs gene has been mapped to UL41 (Kwong et al., 1988) and when this gene is cloned from a HSV-2 strain G into the early shut-off deficient HSV-1 strain 17 efficient early shut-off is restored (M.Fenwick and R.D.Everett, per. comm.).

3. HSV DNA replication

Upon entry into the nucleus of the infected cell the HSV genome is rapidly circularized, possiby by direct ligation of the terminal a sequences (Jacob and Roizman, 1977; Davison and Wilkie, 1983a). Little is known about the mechanism of DNA replication, but the generation of large head-to-tail concatemers, lacking termini, has been interpreted as evidence that at least the later stages of replication may involve a rolling-circle mechanism (Jacob et al., 1979). The concatemers of newly replicated viral genomes are then cleaved within the a sequences between adjacent genomes (Davison and Wilkie, 1981) a process which is tightly linked to the encapsidation of unit length genomes within the nucleus (Ladin et al., 1980; Deiss and Frenkel, 1986).

Although the mechanistic details of HSV DNA replication are largely unknown, all of the cis and trans- acting factors essential for replication are now thought to have been recognized.

3.1 Viral origins of DNA replication

The origins of viral DNA replication have been mapped and characterized, initially by the analysis of replication proficient defective viral genomes (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982) and subsequently with greater accuracy by the characterization of these origins in plasmid replication systems (Stow and McMonagle, 1983; Weller et al., 1985). Three cis-acting regions of the HSV genome have been defined as origins of viral replication. Ori_S is represented twice, being situated between the divergent IE3 and IE4/5 promoters in R_S (Stow, 1982), whilst ori_L is present in a single copy between the divergent promoters of the genes encoding DNA polymerase and the major DNA binding protein (Weller et al., 1985) (see Figure 2). The DNA sequences important for the function of these regions as origins of replication have been defined by supplying HSV replication functions in trans to an ori-containing test plasmid (Stow, 1982, 1985; Stow and McMonagle, 1983).

The ori_L and ori_S sequences show considerable homology; ori_S consists of a 45bp palindromic sequence featuring 18 centrally located A or T residues, flanked by G+C rich sequences (Stow and McMonagle, 1983). Ori_L conserves but extends this palindrome and is highly homologous to ori_S over the 67bp extent of the minimal ori_S sequence (Weller et al., 1985; Quinn and McGeoch, 1985; Deb and Doelberg, 1988). The essential HSV-2 ori_S sequences are very similar to those in HSV-1 (Lockshon and Galloway, 1988). Hubenthal-Voss et al. (1987) have reported that the HSV-1 ori_S is contained within a transcribed open reading frame which could encode a 34kDa protein. The significance of this observation is unknown and McGeoch et al. (1988b) doubt whether this ORF represents a true protein coding sequence.

Viruses with ori_L or one copy of ori_S deleted are viable in cell culture (Polvino-Bodnar et al. 1987; Longnecker and Roizman, 1986). It is unknown whether it is significant that HSV encodes multiple origins for DNA replication or that they are positioned between the divergent promoters of important regulatory and replication genes. Attention is again being focussed on the structure and function of the origins following the demonstration that the product of an essential DNA replication gene binds to sequences in the origin (see below, Elias et al., 1986; Elias and Lehman, 1988; Olivo et al., 1988).

3.2 Viral proteins involved in DNA replication

A number of virus-specified functions involved in DNA replication and metabolism have been identified in virus-infected cells.

(a) A virally encoded DNA polymerase, sensitive to phosphonoacetic acid inhibition and also directing 3' to 5' exonuclease proof-reading activity (Keir et al., 1966; Purifoy et al., 1977; Leinbach et al., 1976; Knopf, 1979).

(b) The major DNA binding protein (ICP8) encoded by HSV is also essential for DNA replication and associates with replicating DNA (Bayliss et al., 1975; Powell et al., 1981; Quinlan et al., 1984). Functional ICP8 is essential for the organization of viral DNA into

pre-replication complexes (de Bruyn Kops and Knipe, 1988).

(c) An origin binding protein has been described (Elias et al., 1986; Koff and Tegtmeyer,1988) and assigned to an essential gene, UL9 (Elias and Lehman, 1988; Carmichael at al., 1988; Olivo et al., 1988).

(d) An essential DNA binding protein, $65K_{DBP}$, has been characterized (Marsden et al., 1987; Marchetti et al., 1988; Parris et al., 1988).

(e) Alkaline exonuclease (Preston and Cordingley, 1982) is an essential virus gene product (Moss, 1986).

(f) Viral-specified ribonucleotide reductase seems to be essential under some growth conditions (Preston et al., 1984; Goldstein and Weller, 1988a,b).

(g) A virally encoded dUTPase is non essential for growth in tissue culture cells, but may be important for virus growth under some conditions (Preston and Fisher, 1984; Williams, 1988)

(h) Virally encoded thymidine kinase (pyrimidine deoxyribonucleoside kinase) is also dispensible for virus growth (Kit and Dubbs, 1963a,b; Jamieson et al., 1974).

(i) A virally encoded uracil-DNA glycosylase DNA repair enzyme has been identified (Caradonna et al., 1987) and assigned to UL2 (Worrad and Caradonna, 1988; J.Mullaney, H.W.McL.Moss and D.J.McGeoch, per. comm.).

(j-l) Virally induced DNA topoisomerase type I, helicase and primase activities have been reported (Muller et al., 1985; Crute et al., 1988; Holmes et al., 1988)

Challberg (1986) has developed a plasmid amplification assay which has allowed the identification of all the HSV-1 genes essential for ori_S directed replication (Challberg, 1986; McGeoch et al., 1988a; Wu et al., 1988). Seven gene products are sufficient to direct plasmid replication on this assay they are the products of the UL genes 5, 8, 9, 29, 30, 42 and 52. All of these genes correspond to loci with known temperature sensitive mutations affecting DNA replication (McGeoch et al., 1988a) and four of them encode proteins with previously characterized replication

functions: UL9 encodes the origin binding protein (Olivo et al., 1988; H.Weir, J.M.Calder and N.D.Stow, per.comm); UL29 and UL30 encode the major DNA binding protein and the DNA polymerase (Quinn and McGeoch, 1985); and UL42 encodes the $65K_{DBP}$ (Marchetti et al., 1988; Parris et al., 1988).

Interestingly none of the DNA metabolism activities listed above (e to i) were essential in this assay. This probably does not provide a true reflection of their importance in vivo, although in rapidly dividing cells cellular functions are probably able to substitute for these viral functions (Goldstein and Weller, 1988b; Williams, 1988). The products of UL5 and 52 have also been shown to be essential for viral DNA replication and late gene expression, but not for early gene expression (Weller et al., 1987; Goldstein and Weller, 1988c; Zhu and Weller, 1988).

4. Virus maturation

The viral a sequences contain all the necessary signals for the site-specific cleavage and packaging of unit length genomes in the nucleus of the infected cell (Stow et al., 1983; Deiss et al., 1986). These two processes are tightly coupled, with cleavage of DNA being a prerequisite for, or occuring concurrently with, the packaging of DNA (Ladin et al., 1980; Vlazny et al., 1982; Deiss and Frenkel, 1986). Although the length of DNA cleaved and packaged is not dependent upon the capacity of the capsids, and shorter molecules can be encapsidated, only nucleocapsids containing approximately unit length DNA are enveloped (Vlazny et al., 1982). Encapsidation is thought to involve the formation of an intermediate capsid, into which DNA is inserted and wound around a protein core to form an electron-dense toroid structure (Perdue et al., 1976). Intranuclear capsids are enveloped in a membrane enriched in viral glycoproteins by budding through the inner lamella of the nuclear membrane into the perinuclear space (Roizman and Furlong, 1974). Viruses are then transported to the cytoplasmic membrane, possibly via the golgi apparatus (Johnson and Spear, 1982) with virus egress typically initiating about eight hours post infection.

SECTION 1C: THE HSV-1 TRANSCRIPTIONAL PROGRAMME

1. Overview of HSV-1 gene expression

Upon lytic infection of tissue culture cells the genes of HSV-1 are expressed in a temporal cascade which can be divided into three main phases, immediate-early (IE or alpha), early (E or beta) and late (L or gamma) (Honess and Roizman, 1974; Clements et al., 1977). In general there is a close correlation between the detection of individual mRNA transcripts and the kinetics of the expression of the proteins encoded by them, indicating that transcriptional control is the major regulatory process occuring during infection (Zhang and Wagner, 1987; Smith and Sandri-Goldin, 1988).

The IE proteins are the first to be detected after infection, within 1h p.a., and their synthesis is shut-off within 3-4h p.a.. The synthesis of the E proteins is detectable shortly after the appearance of the IE proteins, but peaks later at about 4-6h p.a. before being shut-off at late times. Late gene products are not detectable until the onset of DNA replication at around 2h p.a. and they continue accumulating in large amounts at late times without being shut-off. Detailed analysis of viral mRNA synthesis and accumulation indicates that an accurate description of the kinetics of mRNA transcription is more complex than the broad IE, E or L classification (Zhang and Wagner, 1987; Weinheimer and McKnight, 1987) and provides some evidence that post-transcriptional events do have a role in controlling HSV gene expression (Weinheimer and McKnight, 1987; see Section 1C2 below).

Although the initial classification of HSV gene expression was based on the kinetics of viral protein synthesis, it has since been shown to have biochemical significance in that the sequential appearance of these protein classes is regulated in a cascade manner. That is, expression of IE polypetides is essential for the induction of early genes, and the function of early gene products is essential for the expression of late genes. This can be illustrated experimentally by the use of metabolic inhibitors which block viral DNA or protein synthesis

(Honess and Roizman, 1974, 1975; Clements et al., 1977; Jones and Roizman, 1979). The IE genes are the only class which can be expressed upon virus infection in the absence of de novo protein synthesis (Kozak and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979). Whilst the transcription of early and late genes is dependent upon the function of IE proteins, (Honess and Roizman, 1974, 1975) late gene expression is dependent upon viral DNA replication, which is itself a function of viral early gene products (Jones and Roizman, 1979).

HSV late genes vary in the dependence of their expression upon viral DNA replication and their sensitivity to inhibitors of replication. Indeed late genes have been subdivided into true-lates, whose expression is critically dependent on DNA replication, and early-lates, which can be transcribed to some extent in the absence of DNA synthesis (Wagner, 1985; Roizman and Batterson, 1985). Subdivisions have also been applied to early genes to take account of differences in their pattern of expression and shut-off. A useful subclassification is the beta-gamma or "intermediate" genes which are expressed at early times, but accumulate further with DNA replication (Spear and Roizman, 1980).

Figure 3 shows a summary of the factors interacting in HSV gene regulation which will be discussed in more detail in the following sections.

2. IE gene regulation

HSV-1 encodes five IE mRNA species from the five IE genes shown in Figure 2 (IE175, IE110, US1, UL54 and US12). The nomenclature given to the HSV IE genes, transcripts and proteins has become increasingly confused over the years, and is summarized in Table 1. The nomenclature given in the first two columns shall be used throughout this thesis, occasionally using the gene assignations given in column 3 when referring to the genome sequence map (Figure 2).

Analysis of the IE genes by RNA transcript mapping and DNA sequencing has revealed several interesting features of these genes (Clements et al., 1979; Rixon et al., 1982; Murchie and McGeoch, 1982; McGeoch et al., 1985, 1986, 1988b; Perry et al., 1986). Unlike the organization of

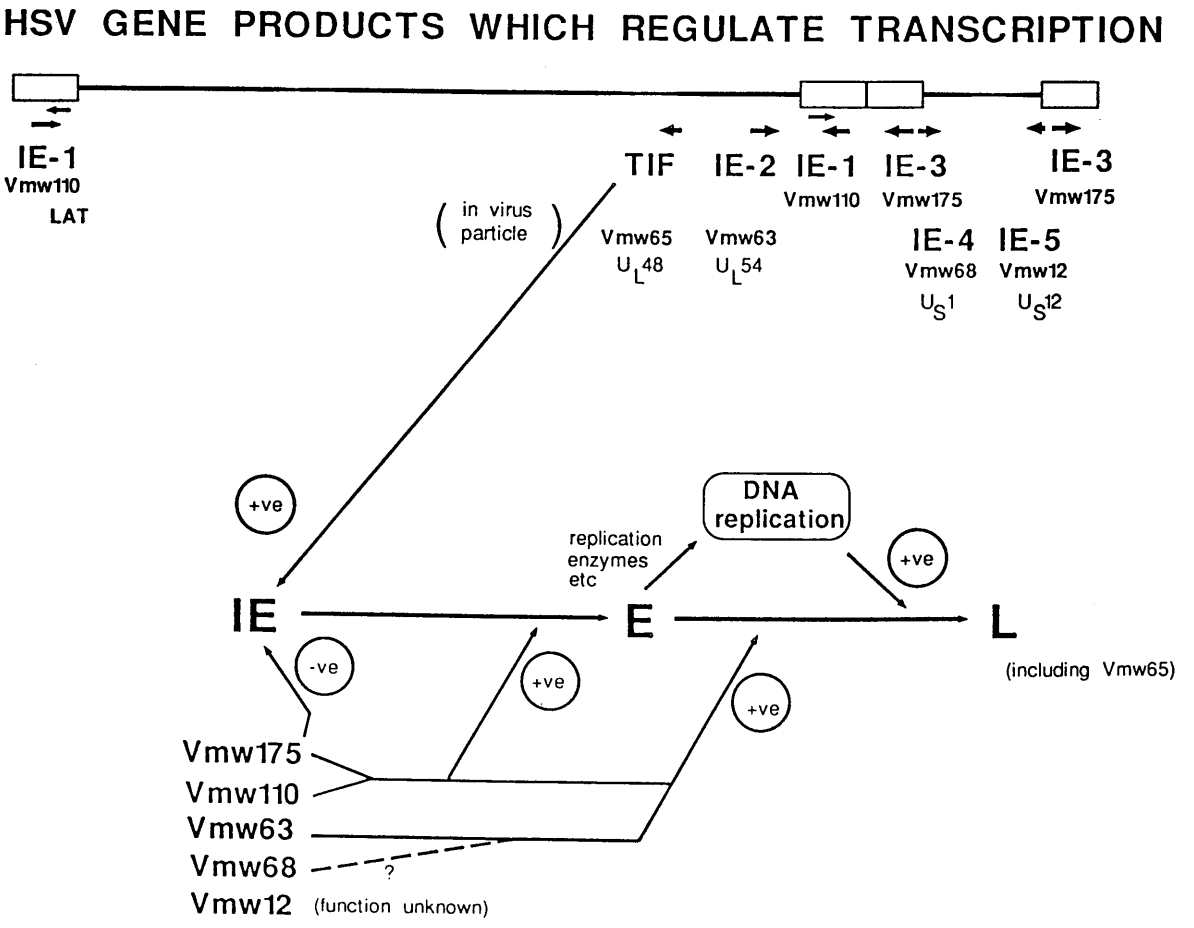


Figure 3: HSV gene products which regulate transcription. (Reproduced from an original diagram by RD Everett). The positions of the genes encoding the 5 IE proteins and the IE transactivating protein, Vmw65, are shown on a map of the HSV-1 genome (see Figure 1 and Table 1). The role of each of these gene products in regulating viral gene expression is shown. Vmw68 may have a role in activating late gene expression. The role of Vmw12 is unknown (see text). Early gene products, in particular those required for DNA replication, are required for late gene expression.

Table 1: Nomenclature given to HSV-1IE genes, transcripts and proteins.

1 Gene	2 Protein (M.Wt.)	3 Gene	4 Protein	5 Gene/ Protein	6 Other names
IE1	Vmw110	IE110	ICP0	alpha 0	IE-0
IE2	Vmw63	UL54	ICP27	alpha 27	IE63
IE3	Vmw175	IE175	ICP4	alpha 4	IE-4
IE4	Vmw68	US1	ICP22	alpha 22	IE68
IE5	Vmw12	US12	ICP47	alpha 47	IE12

Table 1: Nomenclature given to HSV IE genes, transcripts and proteins. Alternative nomenclature for the five IE genes and their products is given. Column 2 lists the nomenclature system used throughout this thesis based on the apparent M.Wt. of the proteins on SDS gels. The Chicago classification of the genes and protein products is given in columns 4 and 5. The IE genes are generally referred to by the simple 1-5 description (column 1) but occasionally referred to by their gene number assignation (see Figure 2; McGeoch et al., 1988b). Some of the more common alternative names used to describe the genes and proteins are given in column 6.

other classes of HSV genes, which seem to be randomly dispersed throughout the genome, the IE genes seem to be localized around the internal and terminal repeats. In fact the genes encoding Vmw110 and Vmw175 map entirely within the R_L and R_S segments respectively, and hence are diploid. The 5' termini of IE mRNAs 4 and 5 are also located within R_S and share common promoter and RNA leader sequences which are spliced to the unique open reading frames US1 and US12 in the U_S region. Splicing is an apparently rare phenomenon in HSV (Wagner, 1985) and so it is interesting that the IE1 transcript is also spliced. That the IE3 and IE 4/5 promoters diverge from the origin of replication in R_S, ori_S, is of unknown significance.

As described above (Section 1A1) the five IE early genes are the first to be expressed upon virus infection, and their transcription is not dependent upon de novo protein synthesis. Indeed it has been shown that the HSV-1 IE genes are transcribed by the unmodified host-cell RNA polymerase II machinery (Costanzo et al., 1977) and that naked, deproteinized DNA is infectious (Graham et al., 1973). However, IE gene transcription is also stimulated by a component of the HSV particle (Post et al., 1981; Campbell et al., 1984, see below).

The IE proteins are first detectable within 1h p.a. and their synthesis is shut-off at 3-4h p.a. However, recent studies using transcription run-on assays in isolated nuclei to measure accurately the rate of gene transcription have shown that this differs markedly from the observed mRNA and protein accumulation (Weinheimer and McKnight, 1987). Indeed, only transcription of the IE3 gene seems to be rapidly turned off (after 1h) whilst the IE1 and 4/5 genes are still transcribed with increasing rates from 1-5h p.a. In contrast the patterns of mRNA accumulation in the presence and absence of protein and DNA synthesis inhibitors are similar for IE genes 1, 3 and 4/5, indicating that a post-transcriptional level of control must be regulating mRNA accumulation.

Several authors have reported that the IE 4/5 mRNA accumulates in a similar kinetic pattern to early mRNA, although beginning earlier and not requiring de novo protein

synthesis (Gelman and Silverstein, 1987a; Zhang and Wagner, 1987; Weinheimer and McKnight, 1987). A novel IE4 transcript has even been reported *in vitro* which was not repressed by Vmw175 (Pizer *et al.*, 1986).

The expression of the HSV-1 IE promoters is regulated by the complicated interplay between cellular transcription factors and at least three viral gene products: the alpha transinducing factor (TIF), Vmw65, which is present in the virion particle, the IE transactivating protein Vmw110 and the multifunctional IE protein Vmw175 which may be involved in both IE transactivation and repression (see for example O'Hare and Hayward, 1985a,b, 1987; Gelman and Silverstein, 1987a,b; and below). These control mechanisms are described in the proceeding sections.

2.1 Structure of IE promoters

The upstream regulatory sequences of the HSV IE genes have been shown to confer IE regulation on chimaeric genes when inserted into recombinant viruses or biochemically transformed cells (Post *et al.*, 1981). All five of the IE promoter/regulatory regions have been dissected and shown to respond to IE regulation in transfection assays (Mackem and Roizman, 1982a,b; Cordingley *et al.*, 1983; Preston *et al.*, 1984) although there are reports that the IE2 promoter behaves more like an early promoter in certain cell-types (Gelman and Silverstein, 1987a).

The promoter regions of the IE genes, like many cellular promoters can be dissected into two components, a minimal promoter which includes a "TATA box" sequence and is necessary for both basal and induced levels of transcription, and upstream regulatory sequences containing multiple *cis*-acting elements conferring enhancer function and responsivity to IE regulation (Mackem and Roizman, 1982a,b; Cordingley *et al.*, 1983; Preston *et al.*, 1984; Kristie and Roizman, 1984; Bzik and Preston, 1986). The upstream regulatory region from -174 to -331 of the IE3 promoter has been shown to act as a constitutive enhancer (Lang *et al.*, 1984; Preston and Tannahill, 1984). However, the distinguishing feature of all the IE upstream regions, at least in transfection assays, is their responsivity to

activation by a component of the virus particle (see Section 1C2.2).

At least five cis-acting elements have been recognized in the promoters, which respond to a variety of viral and cellular trans-acting factors. The minimal promoter regions resemble those of inducible cellular promoters in containing the TATA box element which is bound by an essential factor for RNA polymerase II transcription, TFIID (see Section 1E1). All of the IE promoters contain GC boxes (Sp1 binding sites) and in the case the IE3 and 4/5 promoters these have been shown to bind and respond to Sp1 activation of transcription in vitro (Jones and Tjian, 1985). One to three copies in either orientation of a consensus TAATGARAT (R=purine) are present in each of the upstream regions of the IE genes (within -140 to -340) and functional analyses have shown that this element is essential for the response to transinduction by the virion-associated polypeptide Vmw65 (Mackem and Roizman, 1982b; Preston et al., 1984; Kristie and Roizman, 1984; Gaffney et al., 1985; Bzik and Preston, 1986; O'Hare and Hayward 1987a). Recently another motif present in the IE3 enhancer, GCGGAA, has been implicated in the response to Vmw65, acting in cooperation with TAATGARAT (Triezenberg et al., 1988b). The IE TAATGARAT consensus sequence overlaps with a striking homology with binding sites for a cellular factor NFIII or octamer-binding transcription factor (OTF-1) (Pruijn et al., 1986; Fletcher et al., 1987) and indeed the factor that binds to the TAATGARAT motif appears to have identical specificity to OTF-1 (O'Hare and Goding, 1988; Gerster and Roeder, 1988). The interplay of these sequence elements, their cognate cellular binding factors and the virion transinducing factor is discussed below (Section 1C2.2).

In addition to the presence of sequence motifs involved in the activation of IE gene expression, three of the five IE promoters have copies of the Vmw175 consensus binding site ATCGTC, which has been implicated in the repression of at least the IE3 promoter (Gelman and Silverstein, 1987b; Deluca and Schaffer, 1988). The IE1 and 3 promoters contain perfect copies of this ATCGTC motif and have been shown to bind Vmw175 (Faber and Wilcox, 1986a, 1988; Kristie and

Roizman, 1986a,b; Muller, 1987). The IE2 promoter contains an imperfect match to the ATCGTC motif and may be bound by Vmw175 with somewhat weaker affinity (Kristie and Roizman, 1986a; Faber and Wilcox, 1988). Only the IE3 promoter has been convincingly shown to be repressed by Vmw175 (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b, 1987; Gelman and Silverstein, 1986, 1987a; Roberts et al., 1988). Sequences in the IE3 promoter not containing the ATCGTC consensus have also been reported to bind Vmw175 (Kristie and Roizman, 1986b; Michael et al., 1988).

2.2 Transactivation of IE transcription by the virion associated transinducing factor Vmw65.

A protein component of the HSV virion has been shown to transinduce IE transcription (Post et al., 1981) and has been identified as the product of gene UL48, Vmw65, also known as VP16 or alpha-TIF (Campbell et al., 1984; Dalrymple et al., 1985; Pellet et al., 1985). The activity of this polypeptide is unaffected by UV-irradiation of the virus particles; it is still functional when supplied by superinfection with a virus mutant unable to release DNA from its capsid and seems to be localized in the virus tegument, (Heine et al., 1974; Batterson and Roizman, 1983).

HSV-1 TIF has been shown to transinduce pseudorabies (PrV) virus IE genes, which also conserve TAATGARAT-like motifs in their upstream regulatory sequences (Campbell and Preston, 1987). However, PrV particles do not contain an analagous transinducing factor that functions either on PrV or HSV TAATGARAT elements (Batterson and Roizman, 1983; Campbell and Preston, 1987). VZV has been shown to encode a protein homologous to the HSV TIF (Dalrymple et al., 1985; Davison and Scott, 1986) but this 45kDa open reading frame lacks the carboxy-terminal 80 amino acids of Vmw65 essential for transactivation activity (see below).

It has also been reported that the products of HSV-1 genes UL46 and UL47 may be involved in modulating the activity of Vmw65 (McKnight et al., 1987).

The mechanism by which Vmw65 induces IE genes through the TAATGARAT responder elements has been an area of intensive investigation in recent years. The HSV-1

TAATGARAT elements have been shown to be bound by a factor present in uninfected cells (Kristie and Roizman, 1987; Preston et al., 1988; O'Hare and Goding, 1988; Triezenberg et al., 1988b). This factor is thought to be related to the octamer-binding factor (OTF-1, NFIII or OBP) a previously recognized cellular transcription factor involved in mediating transcriptional activation in a variety of promoters and enhancers (Pruijn et al., 1986; Fletcher et al., 1988; O'Hare and Goding, 1988). Indeed affinity purified OTF-1 and OTF-2 have been shown to bind to TAATGARAT elements, and purified Vmw65 induces formation of an additional (VIC or IEC) complex involving OTF-1. Because the efficiency of complex formation was increased by the addition of cell extract it was suggested that another cell factor may be involved in complex formation, perhaps by interacting with the GARAT portion of the TAATGARAT sequence (Gerster and Roeder, 1988). A second element, GCGGAA, has recently been identified in the IE3 enhancer which binds to an unrelated cellular factor and seems to work in synergy with TAATGARAT and its cognate factor(s) to mediate IE transactivation in response to Vmw65 (Triezenberg et al., 1988a,b; Bzik and Preston 1986).

Vmw65 does not seem to bind to DNA directly (Marsden et al., 1987) but forms a complex (IEC) with the cellular proteins bound specifically to TAATGARAT (Preston et al., 1988). This complex can be assembled in vitro using extracts of infected cells or extracts of uninfected cells combined with Vmw65 purified from virions or synthesized in vitro (Preston et al., 1988; Ace et al., 1988). Mutant forms of Vmw65 incapable of forming IEC in vitro are also unable to activate IE promoter transcription in transfected cell, demonstrating a functional link between complex formation and transactivation (Ace et al., 1988).

Vmw65 would seem therefore to activate transcription in a manner similar to other viral transactivators, such as adenovirus E1a and HSV Vmw110 and Vmw175, which unlike many cellular transacting factors do not bind specific target sequences to mediate their action (Sections 1D1.5 and 1E3.4). The mechanism of Vmw65 transactivation is distinguished, however, by exhibiting target promoter

specificity through its interaction with a cellular factor exhibiting target specificity.

Vmw65 is also an important structural component of the virus particle, but the domains of the protein important for virus assembly are distinct from those involved in transactivation (Ace et al., 1988). Indeed an HSV-1 mutant, in1814, has been constructed which is deficient in IE transinduction, but assembles apparently normal virus particles (Ace et al., 1989). Conversely, an HSV-2 mutant, ts2203, has a lesion within the 5' end of the gene encoding Vmw65 which causes a ts block in virion assembly, but does not affect IE transcriptional induction (Ramsay, 1987; Ace et al., 1988).

It has recently been shown that a region within the 78 amino acid tail of Vmw65 is essential for its transinducing activity, (Triezenberg et al., 1988a). This tail is apparently not involved in IEC formation but in the subsequent activation of transcription, and deletion of the acid tail produces a dominant mutant which blocks the activity of wild-type Vmw65 and virus infectivity (Triezenberg et al., 1988a; Friedman et al., 1988). Like the activating domain of the yeast transactivators GAL4 and GCN4 (Section 1E3.3) it seems to be the acidity of the region per se that is necessary for transactivation. Indeed the acid tail of Vmw65 can substitute for the GAL4 acidic activating domain, without affecting GAL4's target specificity (Sadowski et al., 1988). These acid domain transactivators are thought to activate transcription by interacting with TFIID, the TATA box binding factor and it may be that Vmw65, in the IEC, works by a similar mechanism (Horikoshi et al., 1988a; Lin et al., 1988; Sadowski et al., 1988).

Thus Vmw65 has been shown to have two separate domains involved in transactivation of IE promoters; the first, an internal domain defined by Ace et al. (1988) mediates the indirect association with target promoters through the formation of IEC with a cellular factor bound to TAATGARAT motifs; the second, carboxy-terminal domain seems to be responsible for transactivation of RNA polymerase II transcription by a common mechanism involving the

protein:protein interactions of a highly acidic region of the polypeptide (Triezenberg et al., 1988a; Friedman et al., 1988; Sadowski et al., 1988).

The significance of the HSV TIF to the virus life-cycle is currently a matter for interesting speculation. Recent studies with the non-transinducing mutant show that the transactivation function contributes to virus infectivity in tissue culture and pathogenicity in mice, although not strictly essential for growth in vitro (Ace et al., 1989). This phenotype resembles that of viruses with null mutations in the gene encoding Vmw110 (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989). This may reflect the importance of these two gene products in committing the infecting virus to the lytic infectious cycle, and perhaps the function of Vmw65 has evolved to ensure adequate expression of Vmw110. Indeed it has been observed that in cells infected with in1814 the accumulation of mRNA from only two IE genes, those encoding Vmw110 and Vmw63, is significantly reduced compared to a wild-type infection (Ace et al., 1989). It is interesting that interferon-mediated inhibition of HSV infection in human cells involves the inhibition of IE transcription (Mittnacht et al., 1988; Oberman and Panet, 1988).

Why HSV alone of the alphaherpesviruses seems to have evolved this virion protein-mediated transinduction of IE expression is unknown.

3. Early and late gene expression

The criteria used to define HSV early and late genes are somewhat ambiguous. Early genes require prior synthesis of IE gene products for their expression (Honess and Roizman, 1974; Clements et al., 1977) and respond to IE product transactivation; whilst late genes additionally require DNA synthesis for their expression. Early gene expression begins after the appearance of functional IE products in the infected cell, typically reaching a peak at 4-6h p.a. and may decline at late times of infection (Honess and Roizman, 1974; Wagner, 1985; Weinheimer and McKnight, 1987; Zhang and Wagner, 1987). The expression of late genes is also dependent upon functional Vmw175 (Watson and Clements, 1980)

but is also dependent upon DNA synthesis and is blocked by metabolic inhibitors of viral DNA replication and ts mutations which prevent DNA synthesis at NPT (Swanstrom and Wagner, 1974; Honess and Roizman, 1974; Powell et al., 1975; Marsden et al., 1978; Jones and Roizman, 1979; Holland et al., 1980; Conley et al., 1981; Pedersen et al., 1981). Late gene expression is first detected at around 2-3h p.a., when viral DNA replication is initiated, and late gene products continue to accumulate until 10-16h p.a.. DNA synthesis peaks at around 8h p.a. (Munk and Sauer, 1964; Roizman, 1969; Wilkie, 1973).

There is not always a clear and obvious distinction between each temporal class of HSV gene. For example, the large subunit of ribonucleotide reductase (RR_1) has traditionally been defined as an early gene product, but is expressed very early in infection and accumulates in the absence of IE transactivation (Roizman and Batterson, 1985). The distinction between early and late genes is also somewhat confused, for example, the gene encoding gD is expressed very early in infection and responds to transactivation by IE products, but requires DNA synthesis for maximal expression (Gibson and Spear, 1983; Johnson et al., 1986). VP5, often referred to as a late gene product, is expressed with similar kinetics to gD, but responds to activation by IE products less efficiently than gD (Costa et al., 1985; DeLuca and Schaffer, 1985; Harris-Hamilton and Bachenheimer, 1985; Everett, 1986). Genes exhibiting these kinetics are sometimes referred to as early-lates or leaky-lates. Although the transcription of these early-late genes is increased by DNA replication this may simply be due to an increase in template copy number, and not a direct requirement for DNA replication (Johnson and Everett, 1986a). Furthermore, the expression of these genes generally is decreased at late times of infection by an unknown mechanism (Honess and Roizman, 1974; Johnson et al., 1986).

The synthesis of two classes of transcripts therefore seems to be affected by the inhibition of DNA replication: early-lates, which require replication for maximal expression, and true-lates, whose expression is almost

these elements correspond to well defined cellular promoter elements which are known to interact with cellular transcription factors such as CCAAT-box binding factor and Sp1, and indeed binding by these factors has been demonstrated in the case of the tk promoter (Jones et al., 1985; Graves et al., 1986). The same promoter elements are required for basal and induced levels of transcription in transfected cells and in situ on the virus chromosome (Everett, 1983, 1984b; Eisenberg et al., 1985; El Kareh et al., 1985; Coen et al., 1986). As discussed in Section 1D2 the most important element for IE transactivation of these promoters is the "TATA-box" and its cognate cellular transcription factor TFIID, through which the action of Vmw175 is probably mediated.

The promoter regions required for expression of two true-late genes, US11 and gC, have been dissected in detail, (Johnson and Everett, 1986a,b; Johnson, 1987; Homa et al., 1986, Shapira et al., 1987). The only functionally identifiable element in these promoters is the TATA-box, which functions as a normal true-late promoter when linked to a functional viral origin of replication (Johnson and Everett, 1986a,b; Homa et al., 1986). Although the onset of US11 transcription coincided with the onset of plasmid replication, quantitation of the results suggested that the stimulation of US11 mRNA accumulation was far greater than could be explained by the template copy number (Johnson and Everett, 1986a).

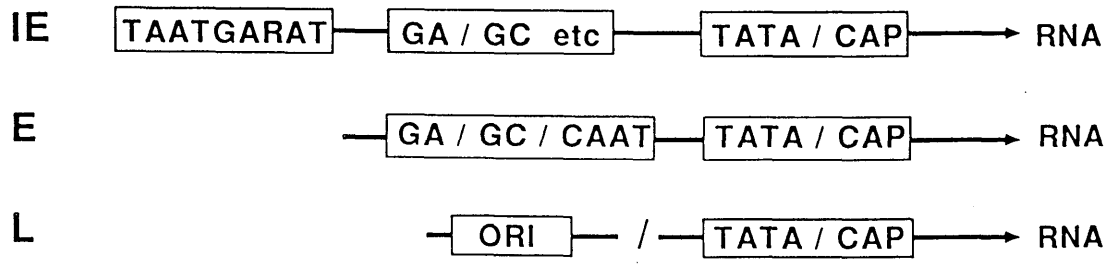
Both the US11 and gC promoters were responsive to IE mediated transactivation in the absence of DNA replication, (Johnson, 1987; Shapira et al., 1987). Furthermore, the early gD promoter could be converted into a true-late promoter simply by deletion of sequences upstream of the TATA-box (Johnson and Everett, 1986b) whilst a similar deletion of the tk promoter apparently abolished its responsivity to IE transactivation (Eisenberg et al., 1985; Shapira et al., 1987). It is unclear why these plasmid-borne late promoters should be responsive to IE transactivation in the absence of DNA replication, whilst promoters resident on the viral chromosome are not efficiently expressed in the absence of DNA replication

(DeLuca and Schaffer, 1985; Shapira et al., 1987; Johnson, 1987; Rice and Knipe, 1988). It has been suggested that the onset of DNA replication may provide a switch for late gene expression, which could for example involve a transient or lasting structural change in the DNA template, or that perhaps part of the replication machinery is needed in situ to initiate late gene transcription.

Recent elegant experiments performed by Mavromara-Nazos and Roizman (1987) confirm the suggestion of Johnson and Everett (1986a) that replication provides an essential cis-acting function for late gene expression. These experiments demonstrated that a true-late promoter on a non-replicating HSV chromosome could not be activated by trans-acting factors specified before or during replication of another HSV virus. This suggests that one of the main requirements for DNA replication in the activation of late promoters may be to alter the DNA template to allow their transcription, perhaps by alleviating some as yet unrecognized negative regulation of these genes. If these changes permitted more promiscuous promoter usage by RNA polymerase (in the absence of upstream regulatory signals) they may explain the increase in apparently uncontrolled symmetric transcription at late times of infection, with an increase in apparently randomly initiated RNA molecules.

It seems therefore that the HSV promoters can be divided into three classes by virtue of their structure, and that this division reflects how these promoters are regulated in a viral infection. These model promoter structures are illustrated in Figure 4. Late gene promoters seem to be characterized by the presence of a TATA-box in the absence of upstream regulatory sequences, and are linked to an origin of replication which substitutes indirectly for upstream activating sequences at late times of infection. Both IE and early promoters contain distal regulatory sequences which are not virus specific and interact with a variety of cellular transcription factors. In addition IE promoters contain far upstream elements responsible for their transinduction by Vmw65 and which in at least some cases acts as enhancer-like elements (see Section 1C2.1).

It is not known whether the decline in early gene



DNA SEQUENCE STRUCTURES OF HSV PROMOTERS

IE PROMOTERS CONTAIN HSV IE SPECIFIC RECOGNITION ELEMENTS

E AND L PROMOTERS DO NOT CONTAIN ANY APPARENT VIRUS-SPECIFIC REGULATORY SEQUENCES

Figure 4: DNA sequence structures of HSV promoters. (Reproduced from an original diagram by RD Everett). Stylized structures are shown for each of the three temporal classes of HSV promoter. All HSV promoters contain a typical TATA box element (binding site for TFIID) but vary in the upstream regulatory sequences. The sole requirement for true late (L) promoters seems to be linkage to a functional origin of replication, whilst IE and early (E) promoters contain a variety of upstream elements including CAAT boxes, GC boxes and GA rich sequences, some of which are known to be bound by a variety of cellular transcription factors (CTF/CBP, Sp1). In addition IE promoters are distinguished by the presence of at least one copy of an HSV specific motif 'TAATGARAT', which is responsive to Vmw65-mediated transactivation of these promoters.

expression at late times of infection is the result of specific repression of these genes or a more general phenomenon. Nor is it fully understood why late genes fail to be expressed in the absence of DNA synthesis. A simple "explanation" of these phenomena may be that whilst early gene promoters are expressed efficiently at early times, under late conditions late gene promoters are expressed far more efficiently, to the exclusion of other promoters. Perhaps as the viral DNA templates replicate, cellular transcription factors essential for early gene expression are titrated out preventing efficient transcription from early promoters. It has been suggested that early and late genes may be subjected to negative regulation of expression. Indeed Costa et al., (1985) reported that sequences from -75 to -125 of the VP5 promoter seemed to mediate negative regulation of this promoter in uninfected cells. Viral gene products have also been implicated in a general negative regulation of expression, including the major DNA binding protein (ICP8) (Godowski and Knipe, 1983, 1985, 1986) and even Vmw175 which has been reported to negatively regulate the gD promoter in stably transformed cells (Arsenakis et al., 1988). It seems probable, however, that the failure to transcribe late genes at early times simply reflects their lack of upstream regulatory elements. It is more problematic to understand how these promoters are turned on by DNA replication, an effect that is not simply explained by an increase in copy number of the viral chromosome upon replication (Johnson and Everett, 1986a; Johnson, 1987).

4. Post-transcriptional regulation of HSV gene expression

Whilst successive studies have failed to provide conclusive evidence of wide-scale post-transcriptional gene regulation in HSV infection, several observations have implicated post-transcriptional mechanisms in the control of mRNA accumulation and translation.

Weinheimer and McKnight (1987) have shown that whilst the IE3 gene appears to be the only IE gene efficiently repressed at the level of transcription, the accumulation of mRNA of all the IE genes is shut-off, corresponding to a shut-off in polypeptide synthesis. Furthermore, whilst at

late times of infection all regions of the genome appear to be abundantly transcribed, and "symmetric transcripts" homologous to all temporal classes of HSV genes are detected, the synthesis of IE and early polypeptides is shut-off at these times (Godowski and Knipe, 1986; Weinheimer and McKnight, 1987).

In general, however, the accumulation of bona fide mRNA for individual genes is found to correlate well with synthesis of the polypeptide gene product. Smith and Sandri-Goldin (1988) have demonstrated that for a variety of early and late genes the steady state levels of mRNA correlate closely with the levels of polysome-associated mRNA and polypeptide synthesis. In contrast to earlier reports (Johnson and Spear, 1984) they found no evidence of post-transcriptional regulation of gD synthesis.

One well characterized example of post-transcriptional control of gene expression has been reported. Synthesis of DNA polymerase seems to be regulated at the level of translation, and although this gene is transcribed efficiently, very few transcripts are found on the polysomes (Yager and Coen, 1988). This control may be mediated by the occurence of a small ORF upstream of the authentic DNA polymerase coding sequence, deletion of which increases translational efficiency (Dorsky and Crumpacker, 1988) or by the secondary structure of the mRNA leader region overlapping with the translational start codon (Yager and Coen, 1988).

Blair et al., (1987) have also discovered regulatory functions mapping to the untranslated 5' mRNA region of the gene encoding Vmw65. Sequences within this leader seemed to contribute towards the stability of the mRNA, in addition to contributing towards virus specific transcriptional activation of the promoter.

These results indicate that although HSV-1 does not seem to possess a general post-transriptional mechanism for regulating gene expression, and furthermore although vhs mediated mRNA degradation is not host specific (see Section 1B2), the translation and stability of individual gene transcripts may contribute to the overall regulation of HSV gene expression.

SECTION 1D: THE HSV-1 IE GENE PRODUCTS AND THEIR FUNCTIONS

1. Properties of the HSV-1 IE gene products

Five IE proteins have been characterized in HSV infected cells whose genes can be expressed upon infection in the absence of *de novo* protein synthesis and whose promoters respond to transinduction by Vmw65 (see Section 1C2). The five IE gene products, their observed molecular weights and their predicted sizes from DNA sequence information are listed in Table 2.

All of the IE proteins with the exception of Vmw12 are nuclear phosphoproteins which can bind to native DNA *in vitro* and are found to be tightly associated with chromatin in the infected nucleus (Pereira *et al.*, 1977; Marsden *et al.*, 1978; Fenwick and Walker, 1979; Hay and Hay, 1980; Ackerman *et al.*, 1984). Thus both their physical properties and their kinetics of expression make them strong candidates for regulators of the viral life cycle, and indeed four of the five IE proteins have now been implicated in viral gene regulation. However, only two of them, Vmw175 and Vmw63, seem to be absolutely essential for viral multiplication in tissue culture (see below, reviewed Everett, 1987a).

The following subsections review the physical and functional properties of the IE gene products, derived primarily from the study of viruses with *ts* and deletion lesions in the IE genes. A more detailed consideration of the role of the IE gene products is given in a subsequent section (1D2).

1.1 Vmw12

Vmw12 is the least characterized IE protein. Its properties are atypical of the IE proteins, being nonphosphorylated and cytoplasmically localized, and its function is obscure (Preston, 1979b; Marsden *et al.*, 1982). Vmw12 is dispensible for growth in tissue culture cells, since HSV-1 and HSV-2 recombinant viruses deleted for IE gene 5 are viable (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

Table 2: The HSV-1 IE gene products.

1 Gene	2 Protein (apparent M.Wt.)	3 No. residues	4 Predicted M.Wt. (kDa)
IE1	Vmw110	775	78
IE2	Vmw63	512	55
IE3	Vmw175	1298	133
IE4	Vmw68	420	47
IE5	Vmw12	88	10

Table 2: The HSV IE gene products. The IE genes and protein products are listed in columns 1 and 2 by the system of nomenclature used throughout this thesis (see Table 1). Column 4 records the number of amino acids in the predicted polypeptide sequence, and column 4 the unmodified M.Wt. predicted from the amino acid sequence (McGeoch et al., 1988b).

1.2 Vmw68

A virus with a deletion in IE gene 4 has been constructed which removes a region encoding the carboxy-terminal third of Vmw68 (Post and Roizman, 1981). Although this virus was viable in tissue culture it was impaired in late gene induction and showed poor growth at low multiplicities of infection in some cells. It also exhibited reduced neurovirulence in mice (Sears et al., 1985).

Vmw68 would therefore appear to have a role in late gene regulation, at least in some cell types, and perhaps substitutes for a cell type retricted host factor. However, a more complete study of this gene is required to determine whether it is truely non-essential, and whether the carboxy-terminal truncated Vmw68 retains any activity.

1.3 Vmw63

Vmw63 is essential for virus growth in tissue culture. Viral mutants containing ts lesions in the Vmw63 gene show reduced expression of early-late genes and fail to accumulate late gene products at the non-permissive temperature (Sacks et al., 1985). Vmw63 does not seem to be required for the induction of early gene expression and these mutants are able to replicate DNA at NPT. Furthermore, Vmw63 does not seem to transactivate the majority of early promoters in transient assays but does specifically stimulate expression from certain delayed early and late promoters (Everett, 1986; Rice and Knipe, 1988; Sekulovich et al., 1988).

The ts mutants of Vmw63 overexpress Vmw175 and Vmw63 but not Vmw110 at the NPT, suggesting that this protein may be directly or indirectly involved in the repression of IE expression (Sacks et al., 1985). At the NPT these mutants produce an electrophoretically abnormal form of Vmw175 (Sacks et al., 1985; Rice and Knipe, 1988).

Deletion mutants of HSV-1 have recently been constructed which express no Vmw63. These viruses are replication incompetent on non-complementing cell-lines, overexpress some early genes and fail to induce normal levels of DNA replication and true-late gene expression (McCarthy et al., 1989). Vmw63, therefore, has an essential role in

regulating early and late gene expression and possibly regulating IE gene expression. Viruses with ts mutations in Vmw63 have a similar phenotype to a subclass of ts mutants in Vmw175 which are permissive for early gene expression and DNA synthesis, but underproduce late genes at NPT (DeLuca et al., 1984). These results imply that Vmw63 and Vmw175 might function together in regulating late gene expression. Indeed in transfection assays Everett (1986) found that Vmw63 only contributed to the activation of the late VP5 promoter in the presence of Vmw110 and Vmw175, although Vmw63 does seem to be capable of the independent transactivation of the gB promoter (Rice and Knipe, 1988). It has recently been reported that in certain situations Vmw63 can repress Vmw110 and Vmw175 activated expression of a variety of promoters, but activates certain delayed-early promoters in combination with Vmw110 and Vmw175 (Sekulovich et al., 1988). Furthermore repression and activation appear to be separable functions of the Vmw63 polypeptide.

Intriguingly MacLean and Brown (1987) have reported a viable HSV-1 variant which fails to produce detectable amounts of Vmw63 at IE times of infection. However, there is no evident lesion in the IE2 gene and perhaps sufficient Vmw63 is expressed at early times to account for the normal late gene expression induced by this virus.

1.4 Vmw110

No conditional-lethal mutants have been isolated in IE gene 1 and Vmw110 does not appear to be absolutely essential for virus growth in tissue culture. Reduction of Vmw110 expression to less than 10% normal levels by expressing antisense IE1 mRNA in infected cells had little effect on virus gene expression or virus yield (Sandri-Goldin et al., 1987). Virus mutants with large deletions within the Vmw110 coding sequence have been constructed and found to be viable in tissue culture, although they grew poorly at low multiplicities of infection (Stow and Stow, 1986; Sacks and Schaffer, 1987). In common with the Vmw65 transinduction-deficient mutant described in Section 1C2.2 (Ace et al., 1989) the Vmw110 deletion mutant has a high particle to infectious pfu ratio, and seems to have a defect

in initiating virus replication at low multiplicities of infection. This deficiency is not seen at higher multiplicities, when viral gene expression and DNA replication are qualitatively and quantitatively similar to wild-type (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989).

The discovery that Vmw110 was inessential at least under some growth conditions was unexpected because Vmw110 has been shown to be a promiscuous transactivator of both viral and heterologous promoters, acting both independently and in synergy with Vmw175 (Everett, 1984b, 1986; O'Hare and Hayward, 1985a,b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985a). The role of Vmw110 as a transactivator is discussed in greater detail below (Section 1D2.1), and a detailed analysis of its structure and function in Section 1D2.3.

The role of Vmw110 during virus infection therefore is an interesting and unsolved question. Vmw110 clearly is a potent activator of gene expression and confers a strong growth advantage on the virus, without being absolutely essential for growth in tissue culture. Furthermore, the functions of Vmw110 cannot substitute for the essential activities of Vmw175 in viruses carrying deletions in Vmw175 (DeLuca and Schaffer, 1985, 1988; Russell *et al.*, 1987b; this thesis). It would seem therefore that Vmw110 might be important in the activation of viral gene expression in certain cell types, which perhaps are lacking in certain transcription factors. This function might be particularly important at very early times of infection and at low multiplicities of infection in order to ensure adequate Vmw175 or early gene expression and therefore commit the virus to the lytic cycle, (Stow and Stow, 1986; Everett, 1989). It is interesting that viruses deficient in Vmw110 expression cannot reactivate latent HSV in an *in vitro* latency system, suggesting that Vmw110 is necessary to induce the lytic cycle (Russell *et al.*, 1987b).

1.5 Vmw175

(i) Genetic analysis of the function of Vmw175

The isolation and characterizatin of viruses with *ts*

lesions in Vmw175 showed that this function has an essential role in the HSV transcriptional programme. These early ts mutants were found to overproduce IE gene products and fail to express early and late genes at NPT (Marsden et al., 1976; Courtney et al., 1976; Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980). Functional Vmw175 was shown to be continuously required for early gene transcription and IE repression by performing temperature shift experiments with ts-Vmw175 mutants (Watson and Clements, 1980; Dixon and Schaffer, 1980).

The isolation of a class of mutants permissive for early gene expression and a degree of DNA replication suggested that Vmw175 may possess a distinct function required for late gene expression (DeLuca et al., 1984). Indeed the DNA lesions in this class of mutants were mapped to the carboxy-terminal end of the coding sequence and may indicate that this function may be encoded by a distinct domain of the protein. On the other hand the DNA lesions in Vmw175 mutants with extremely "tight" phenotypes, non-permissive for early expression and DNA replication, have been mapped to more amino-terminal regions of the protein, including the tsK mutant which is caused by a single substitution at amino acid 475 (Dixon and Schaffer, 1980; DeLuca et al., 1984; Preston, 1979a; Davison et al., 1984).

Viruses containing deletion or truncation mutants in IE gene 3 have been constructed which have to be propagated on a complementing cell line (DeLuca et al., 1985; Deluca and Schaffer, 1988). The analysis of these mutants will be considered in Section 1D2.4.

(ii) Physical properties of Vmw175

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

Although the sequence of the gene reveals an uninterrupted open reading frame encoding a protein of 1298 amino acids with predicted molecular weight of 133kDa, the observed size of the modified protein recovered from infected cells is 175kDa on SDS polyacrylamide gels (McGeoch et al., 1986, see Table 2). In common with other IE

proteins Vmw175 is post-translationally modified by phosphorylation, but also by poly(ADP)ribosylation *in vitro* (Preston and Notarianni, 1983). Three electrophoretic forms of Vmw175 are distinguishable by gel electrophoresis and all are phosphorylated (Pereira *et al.*, 1977). Threonine and serine residues are the major sites of phosphorylation (Faber and Wilcox, 1986b) and phosphate appears to cycle on and off the protein throughout infection, perhaps altering its affinity for DNA (Wilcox *et al.*, 1980).

Some *ts* mutants of Vmw175 do not seem to be poly(ADP)ribosylated at the NPT (Preston and Notarianni, 1983, Faber and Wilcox, 1986b) and some mutant proteins fail to localize in the nucleus at the NPT, and even interfere with the nuclear localization of other proteins, such as Vmw110 and the MDB protein (Preston, 1979b; Knipe and Smith, 1986).

The pattern of nuclear localization of Vmw175 alters throughout the course of infection. At early times Vmw175 is distributed diffusely throughout the nucleus, whilst at late times it colocalizes with the MDB protein in intranuclear, globular replication compartments (Randall and Dinwoodie, 1986; Knipe *et al.*, 1987).

Whilst most of the IE proteins have been shown to bind to native calf thymus DNA *in vitro* (Hay and Hay, 1980) Vmw175 is the only one known to interact with specific target sequences. A combination of filter binding, gel retardation, DNAse and chemical footprinting techniques have been used to investigate this binding, and a consensus DNA sequence present and protected in all the high affinity binding sites characterized so far has been derived:

nnATCGTCnnYnCCGRCnnCRYCR

with the hexanucleotide ATCGTC considered to be the most important invariant element (Beard *et al.*, 1986; Faber and Wilcox, 1986a, 1988; Muller, 1987; Roberts *et al.*, 1988). Vmw175 has been shown to bind to this sequence in the promoter regions of the IE1, IE3 and gD genes (Beard *et al.*, 1986; Faber and Wilcox, 1986b, 1988; Kristie and Roizman, 1986a,b; Muller, 1987) and also to several viral promoter fragments which lack this sequence, although this binding may be of lower affinity (Kristie and Roizman, 1986a;

Michael et al., 1988). The ability of Vmw175 to bind to a copy of the ATCGTC target sequence at the transcriptional start site of IE gene 3 has circumstantially been implicated in the ability of Vmw175 to repress transcription from this promoter (Muller, 1987; DeLuca and Schaffer, 1988; Roberts et al., 1988). The functional significance, if any, of the upstream IE1 Vmw175 binding site is not known (Roberts et al., 1988).

Less is known about the significance of Vmw175 DNA binding in the activation of HSV and heterologous promoters, although the same protein domain is involved in transcriptional activation, repression and DNA binding (DeLuca and Schaffer, 1988; this thesis). The Vmw175 binding site in the gD promoter lies outwith the sequences required for transactivation by IE products (Everett, 1983). Indeed the majority of responsive promoters lack sequences closely related to the consensus binding site, and there is no evidence for any transactivation specific promoter sequences required for this action of Vmw175 (Everett, 1984a; Eisenberg et al., 1985; El Kareh et al., 1985; Coen et al., 1986). This will be discussed in greater detail in Section 1D2.1.

Although the binding of Vmw175 to DNA had previously been reported to be dependent on host cell factors (Freeman and Powell, 1982) recent reports that purified Vmw175 can bind to specific target sequences indicate that the protein can bind to DNA directly (Michael et al., 1988).

(iii) Structure of Vmw175

The sequence of the gene encoding Vmw175 reveals a number of interesting features in the predicted amino acid sequence and structure (McGeoch et al., 1986). The IE gene 3 coding region has the extraordinary base composition of 81.5% G+C and a correspondingly abnormal amino acid composition in the predicted primary structure. The protein is particularly rich in alanine, proline, glycine and arginine residues, together comprising 54.9% of all amino acids. Analysis of the codon usage in the reading frame suggests that the gene has evolved to maximize its G+C content, and indeed amino acids with G+C rich codons would

appear to have been evolutionarily favoured. One prediction of these observations is that the structure of regions of the protein particularly rich in these amino acids may not be critically important for function of the protein (McGeoch et al., 1986).

A less extreme G+C content is found in the VZV gene ORF62, which encodes the homologous protein "IE"140K, and whose DNA sequence is 64.1% G+C (Davison and Scott, 1986). The VZV gene encodes a protein of 1310 amino acids and predicted M.Wt. 140kDa, which probably corresponds to the observed IE protein of 175-185kDa (Shiraki and Hyman, 1987; Felser et al., 1988). Expression of this protein in infected, transfected or biochemically transformed cells complements HSV-1 ts and deletion mutants in Vmw175 (Felser et al., 1987, 1988).

The predicted amino acid sequences of Vmw175 and VZV140K have been compared in detail and found to be highly homologous in three regions: A, B and C (McGeoch et al., 1986, Davison and McGeoch, 1986; see Figure 5). These regions are also conserved in a third alphaherpesvirus IE protein, the PrV IE180 protein (C.Vlcek, M.Schwyer and V.Paces, per.comm.). On the basis of this sequence homology between the HSV-1 and VZV proteins the Vmw175 protein has been divided into 5 structural regions, with regions 2 and 4 containing the main regions of homology with the VZV protein (McGeoch et al., 1986; see Figure 5).

The HSV type 1 and type 2 Vmw175 proteins are functionally interchangeable, and have been shown to be colinear and homologous by generating intertypic recombinant chimaeras of the type 1 and type 2 proteins (Smith and Schaffer, 1987a,b).

Little of Vmw175 exhibits any marked predicted secondary structure; this is discussed further in Chapter 4. Region 1 of the protein (residues 1-314) would appear to have an ill-defined structure of "random coil" and is not closely conserved between the three proteins except for a short serine rich tract from residues 186-204 (homology region A).

Region 2 (315-484) is closely conserved between the three proteins, having 35-45% identity between all three and a region of more than 80% identity between VZV and HSV.

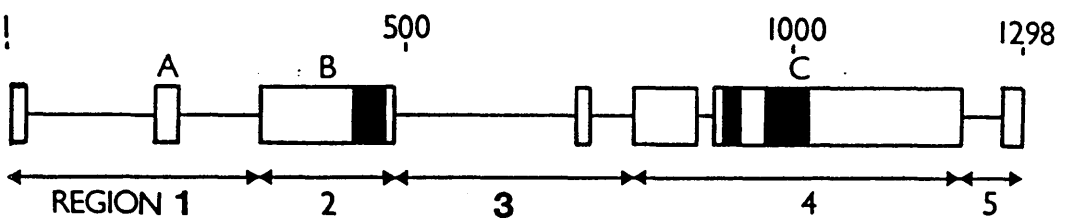


Figure 5: Structure of the protein Vmw175 in relation to the VZV homologue, VZV 140K. Vmw175 has been divided into 5 regions, 1-5, on the basis of sequence and homology (McGeoch et al., 1986). Amino acid residues are numbered 1-1298 from amino to carboxy terminus, and the three main homology regions (A,B and C) are marked. Clearly homologous sections are shown as boxes, with the most homologous sections filled (at least 16 residues identical out of 20). The scale is shown in amino acid residues.

Significantly the single amino acid substitution responsible for the phenotype of the tsK mutation maps in this area at residue 475 (Davison et al., 1984).

Region 3 (485-796) is essentally non-conserved between Vmw175 and VZV140K apart from a short basic amino acid run conserved in both proteins and implicated in the nuclear localization of Vmw175 (DeLuca and Schaffer, 1987, 1988; this thesis). Vmw175 is 214 residues longer than the VZV homologue in this region.

Region 4 (797-1224) is clearly homologous between all three proteins, and has regions of near identity between the VZV and HSV proteins, around which two ts lesions have been mapped (Preston, 1981).

Region 5 (1225-1298) is largely non-conserved. Interestingly, however, both the amino and carboxy terminals of Vmw175 are weakly conserved in VZV140K, although the region of the VZV protein that corresponds to the carboxy-terminal region of Vmw175 is not at the carboxy-terminus.

The main aim of the work presented in this thesis was to examine whether this structural map of Vmw175, shown in Figure 5, has any functional significance, and indeed whether the regions most conserved between the HSV-1 and VZV proteins correspond to regions important for functional activity.

2. Transcriptional regulation by the HSV IE gene products

2.1 Transactivation

(i) Early characterization of the phenomenon of transactivation

Genetic studies have shown that Vmw175 expression is required for early and late gene expression; that Vmw63 has a role in the activation of a subset of viral (late) genes and that Vmw68 may also have a less critical role in activating subsets of viral genes (Section 1D1 above). The transcriptional control of early and late genes has since been investigated by a variety of reductionist approaches in order to define the viral factors responsible for their transactivation and the promoter sequences required for this response. Early studies made use of biochemically

transformed cell lines and demonstrated that integrated copies of HSV early and late genes could be transactivated by superinfection with HSV, and that this transactivation was dependent upon Vmw175 expression. However, in these experiments integrated late promoters behaved with early gene kinetics (Leiden et al., 1976; Kit et al., 1978; Sandri-Goldin et al., 1983; Dennis and Smiley, 1984; Silver and Roizman, 1985; Mosca et al., 1985). A few cellular genes can be induced by HSV infection, some of which are activated in a Vmw175 dependent fashion (Latchman et al., 1987; Kemp and Latchman, 1988). The transcription of most cellular genes, however, is shut-off upon infection (see Section 1B2).

Everett (1985) showed that heterologous cellular globin promoters introduced into the cellular genome by stable transformation also responded to IE mediated transactivation upon HSV infection, or transfection with plasmids expressing IE gene products, indicating that no viral specific promoter sequences were necessary. Furthermore, when the rabbit beta-globin promoter is introduced into the HSV genome it is regulated upon infection as a typical viral early gene (Smiley et al., 1987) although a transduced rodent aprt gene was not activated upon infection (Tackney et al., 1984). Significantly this "housekeeping" gene lacks a TATA box motif in its promoter (Dush et al., 1985; Nalbantoglu et al., 1986).

Stable, biochemically tranformed cell-lines expressing wild-type or ts Vmw175 have also been used to investigate the phenomenon of transactivation (Persson et al., 1985; Persson and Bacchetti, 1987; Arsenakis et al., 1988). Upon HSV infection of such cell lines, in the absence of de novo protein synthesis, endogenously expressed Vmw175 was able to activate the expression of some early genes but not late or some delayed early genes (Persson et al., 1985). Vmw175 expressed in similar cell-lines was also able to transactivate adenovirus early genes when infected with an Ela^{-} mutant, showing that HSV Vmw175, like other herpesvirus IE transactivators, can substitute for Ela activity (Tremblay et al., 1985). Attempts to stably cotransform cell lines with the gene encoding Vmw175 and HSV late genes

have generated complicated results, which may indicate that some HSV early or late genes could be negatively regulated by Vmw175 in special circumstances (Arsenakis et al., 1988).

Practical difficulties in creating stably transformed cell lines and theoretical questions about the nature of the integrated genes have led many investigators to develop short-term transfection assays for IE function.

(ii) Characterization of transactivation by IE products in transient transfection experiments

Short-term transfection assays have been used to dissect the functions of the various IE gene products in great detail. Initial experiments showed that HSV IE proteins caused transcriptional activation of plasmid-borne viral early promoters transfected into tissue culture cells and superinfected with HSV (Everett, 1983; O'Hare and Hayward, 1984). Certain plasmid-borne cellular and adenovirus promoters were found to be similarly responsive to HSV or PrV IE transactivation (Everett, 1983; Green et al., 1983; Imperiale et al., 1983). Cotransfection of cloned IE genes with plasmid-borne reporter genes identified two HSV IE proteins involved in this transactivation (Everett, 1984b). These two HSV IE gene products Vmw110 and Vmw175 have been shown to transactivate a variety of cotransfected HSV early promoters by measuring the accumulation of mRNA or CAT enzyme activity produced from a promoter CAT fusion reporter gene (Everett, 1984b, Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a,b; Gelman and Silverstein, 1985, 1986). Vmw110 and Vmw175 have both been shown to be capable of independent transactivation of transcription, however, in some circumstances the effect of each gene product alone is small, whilst when supplied together they have a synergistic effect upon transcription (Everett, 1984b, Quinlan and Knipe, 1985). Some investigators report strong independent transactivation by each protein whilst not observing the synergistic cooperation (O'Hare and Hayward, 1985a; Gelman and Silverstein, 1985). These differences are probably explained by differences in experimental detail and Everett (1988b) has reported that cell type, growth medium and transfection protocol, as well as promoter sequence, can

effect the relative transactivation by Vmw110 and Vmw175.

Alleles of the gene encoding Vmw175 with ts lesions have been cloned and their activity in transfection assays has been shown to reflect the phenotype of the mutation in the parental virus (DeLuca and Schaffer, 1985).

The characterization of Vmw110 as a promiscuous transactivator of transcription in the transient transfection assays does raise an interesting paradox: why is the transactivating activity of Vmw110 insufficient to replace Vmw175 function in Vmw175 deficient viruses? It has been suggested that mutant alleles of Vmw175 are transdominant and that the mutant protein interferes with the function of wild-type Vmw175 or with other transactivators (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986). It has also been observed that some ts-Vmw175 mutations interfere with the nuclear localization of Vmw110 and other proteins (Preston, 1979b; Gelman and Silverstein, 1986; Knipe and Smith, 1986). Recently, however, viruses with large deletions in IE gene 3 have been constructed which express little or no Vmw175. These viruses are unable to grow on non-complementing cell lines; the viruses are able to express other IE proteins on infection but do not induce early gene expression, indicating that Vmw110 cannot substitute for Vmw175 even in the absence of interfering Vmw175 polypeptide (DeLuca et al., 1985; Russell et al., 1987b; DeLuca and Schaffer, 1988; this thesis).

It is clear therefore that Vmw175 must perform certain functions for which Vmw110 cannot substitute, and although the exact role of Vmw110 in a normal infection is somewhat opaque, it does seem to be important at low multiplicities of infection (see Section 1D2.3). Differences in gene and template dosage in transfected cells compared to a normal infection might explain why Vmw110 can apparently transactivate early promoters in transfection assays but not induce early gene expression in a virus infection. It is also possible that while Vmw175 might increase the rate of transcriptional initiation, Vmw110 might act at a later stage. This could explain the synergistic effect, and the absolute requirement for Vmw175.

The product of IE gene 2, Vmw63, has also been shown to transactivate a subset of viral genes in transfection assays (Everett, 1986; Rice and Knipe, 1988). Vmw63 has been shown to stimulate the activation of certain delayed early and late promoters in combination with Vmw110 and Vmw175 (Everett, 1986; Sekulovich et al., 1988) but to be capable of independently activating the late gB promoter (Rice and Knipe, 1988). These results are consistent with the phenotype of viruses with ts lesions in Vmw63, which fail to express a subset of late genes at NPT (Sacks et al., 1985).

(iii) Promoter elements involved in IE protein transactivation: a model for the mechanism of transactivation.

The DNA sequences required for virus early and late promoter activity have been dissected by recombinant DNA techniques and assayed in transient transfection assays. Two early promoters in particular have been studied in detail, those for the genes encoding gD and tk. No virus-specific sequences required for viral transactivation have been detected in either of these promoters, and indeed the promoter sequences essential for basal and cis-activated transcription are identical to those required for transactivated expression (Everett 1983, 1984a,b; El Kareh et al., 1985; Eisenberg et al., 1985). Various tk promoter mutants have been reintroduced into the viral chromosome and confirm that the same promoter elements are important for activity in the virus and in the transfection experiments (Coen et al., 1986). Significantly, an intact TATA box seemed to be quantitatively more important than other elements in the promoter for activity in the infected cell. A novel mutation-sensitive site was detected just downstream of the tk transcriptional start site and it was suggested that this region might also be important for interactions with the TATA box binding factor-associated pre-initiation complex (Coen et al., 1986). Deletion of all the sequences upstream of the tk promoter TATA-box, however, apparently abolishes its response to IE transactivation although the greatly reduced basal level of expression may in fact make transactivation difficult to measure properly (Eisenberg et

al., 1985; Shapira et al., 1987). On the other hand the true late promoters from the gC and US11 genes seem only to require a TATA-box element to respond to Vmw110 and Vmw175 mediated transactivation (Johnson, 1987; Shapira et al., 1987).

These results, together with the observation that cellular promoters with TATA boxes can be activated by HSV IE products in biochemically transformed cells and recombinant viruses [see above (i)] suggest that the IE transactivator proteins function through common cellular promoter elements, perhaps even the TATA box element itself, which is a fundamental component of most type II promoters. Recent studies by Everett (1988b) have confirmed that changes in the TATA box sequence can alter the efficiency of promoter activation by the HSV IE products, and that some TATA boxes seem to be more responsive to transactivation than others, in certain circumstances.

This preference for certain TATA box sequences over others may reflect a direct interaction between IE proteins and this sequence or, alternatively, that a family of different cellular TATA box binding factors are involved in interacting with a variety of TATA box sequences, and that HSV IE gene products recognize these cellular factors with varying efficiencies. It seems probable that transactivation is commonly mediated through the TATA box, or its cognate binding factor TFIID, both by non-specific viral transactivators such as E1a (Wu et al., 1987; Simon et al., 1988) and cellular transactivators such as ATF and the yeast transactivator GAL4 which have been shown to interact with TFIID to activate transcription (Horikoshi et al., 1988a,b; Hai et al., 1988; Lin et al., 1988).

Partially purified Vmw175 has been shown to transactivate the transcription of a variety of early and late genes in vitro, indicating that its effect on gene regulation is direct (Beard et al., 1986; Pizer et al., 1986). Only one of the promoters tested, gD, contained a Vmw175 binding site, but this sequence has previously been shown to lie outwith the minimal region of the promoter responsive to transactivation (Everett, 1983, 1984a). Presence of this Vmw175-DNA binding site seems to play a

minor role in the activation of gD promoter by Vmw175 in transfection assays and *in vitro* (Tedder and Pizer, 1988).

The closely homologous PrV IE protein IE180 has also been shown to stimulate transcription *in vitro*, and its direct involvement was demonstrated by using a *ts* mutant of the protein (Abmayr *et al.*, 1985). Temperature shift experiments with this *ts*-IE180 protein have shown that transactivation functions at the level of initiation and that inactivated *ts*-IE180 can compete with Ela to inhibit the activation of class II promoters (Ahlers and Feldman, 1987a,b). Vmw175 also seems to exert its effect *in vitro* on the rate of initiation of transcription (Beard *et al.*, 1986) and evidence exists that Vmw175 and Ela share a common mechanism or at least a common cellular component for transactivation. Whilst Vmw175 can substitute for Ela function in activating adenovirus early genes, the presence of Vmw175 interferes with Ela activation, perhaps indicating that they are competing for common cellular factors (Tremblay *et al.*, 1985).

Recent studies with partially purified PrV IE180 protein have shown that this protein stimulates transcription *in vitro* by increasing the efficiency or rate of TFIID binding to the DNA template to form the preinitiation complex (Abmayr *et al.*, 1988). PrV IE180 can potentiate TFIID DNA binding to the template promoter in competition with the assembly of the DNA template into nucleosomes by purified histones *in vitro*. This suggests a mechanism by which viral IE proteins may potentiate expression from infecting viral genomes of naked DNA, by assisting the rapid formation of preinitiation complexes on promoters before they are assembled into nucleosomes, which may preclude promoter recognition (Workmann *et al.*, 1988). Transcriptional activation by the PrV IE180 protein and the adenovirus Ela protein has been shown to function through the same (TATA box) sequences in the adenovirus Elb promoter, suggesting that Ela and IE180 mediate transactivation through a common cellular intermediate which interacts with the TATA box (Wu *et al.*, 1987; Wu and Berk, 1988). Presumably Vmw175 functions by a very similar mechanism to PrV IE180 as the important regions of the protein are highly conserved

between PrV and HSV (Vlcek, Schwyzer and Paces, per.comm.; Sections 1D1.5 and 4.2).

How Vmw110 and Vmw175 might interact at a physical level with components of a responsive promoter is unknown. Both proteins are known to bind DNA (Hay and Hay, 1980) although in the case of Vmw110 it is not known whether this binding is direct or mediated through other cellular or even viral factors. Vmw110 can activate gene expression by itself in the absence of its carboxy-terminal DNA-binding domain, but requires this region to function in synergy with Vmw175 (Everett, 1987b, 1988a). Thus Vmw110 might function in two different ways in the presence and absence of Vmw175.

In contrast the regions of Vmw175 involved in site-specific DNA binding, transactivation and repression are not separable (DeLuca and Schaffer, 1988; this thesis) and DNA binding may be a prerequisite for transactivation. This would appear not to involve site-specific DNA binding to consensus ATCGTC target sites, which are not commonly found in responsive promoters, but may involve a second DNA binding activity, perhaps of lower affinity, to unrelated sites or degenerate ATCGTC sites. Indeed binding to unrelated sites in IE promoters and the 5' untranslated regions of late genes has been reported (Kristie and Roizman, 1986a; Michael et al., 1988).

These somewhat preliminary results would support a model for Vmw175 function in which Vmw175 is involved in activating or stabilizing the formation of pre-initiation complexes, or catalyzing their conversion to initiation complexes, perhaps through an interaction with TFIID.

2.2 Repression of IE gene expression

Viruses with ts lesions in Vmw175 overexpress the IE gene products at the NPT, and functional Vmw175 is required continuously for the repression of IE expression (Preston, 1979a, Dixon and Schaffer, 1980, Watson and Clements, 1980). During the course of a normal virus infection the repression of IE gene transcription is not completely effective and IE RNAs persist until late in infection (Harris-Hamilton and Bachenheimer, 1985). Furthermore, there is evidence that only IE gene 3 is repressed at the level of transcription,

although post-transcriptional mechanisms may control the accumulation of other IE transcripts (Weinheimer and McKnight, 1987).

Genetic evidence exists that the major DNA binding protein (ICP8) functions as a negative regulator of viral gene expression, and that ts mutants in this protein cause an overexpression of Vmw175 at the NPT (Godowski and Knipe, 1983, 1985, 1986). However, it is unclear whether or not this is a specific gene regulation function. The virion associated host shut-off function may also be involved in regulating IE expression at a post-transcriptional level by destabilizing IE mRNA (Fenwick and Owen, 1988).

Plasmids expressing cloned Vmw175 have been shown to repress basal and cis- and trans-activated levels of transcription from the IE3 promoter in a dosage dependent manner (O'Hare and Hayward, 1985b, DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986). Gelman and Silverstein (1987a) have conducted an exhaustive study into the response of each of the four IE promoters to different combinations of Vmw110 and Vmw175. The response was found to be dependent upon the dosage of each effector protein and also upon cell type to a certain extent.

The IE2 promoter was found to behave abnormally in one cell type in that it responded in the manner of an early promoter, and was not repressed. Whereas Vmw110 alone activated all of the promoters, Vmw175 alone had little effect on the IE4/5 promoter and repressed the IE1 and IE3 promoters. The combination of Vmw110 and Vmw175 gave a complicated response dependent upon the gene dosage, stimulating expression more at low concentration than at high concentration.

The interpretation of such experiments is fraught with difficulties and one is never sure how relevant such experiments are to the situation occuring in an infected cell where the number of templates is much smaller. However, it is clear that regulation of the IE promoters is critically dependent upon the relative abundance of Vmw110, Vmw175 and Vmw65 in the infected cell at IE and early times of infection (O'Hare and Hayward, 1985a,b, 1987; Gelman and Silverstein, 1985, 1986, 1987a,b).

Evidence for repression by Vmw175 has only been reported for the IE3 and IE1 promoters, both of which have good matches to the ATCGTC motif in their promoter. Roberts et al., (1988) have demonstrated a direct correlation between the Vmw175 binding site at the IE3 cap site and repression of this gene, but have failed to find any functional significance for the upstream IE1 site. The IE2 promoter does have a degenerate match to this consensus, but is only marginally repressed in one of the two cell types tested by Gelman and Silverstein (1987a). Both the IE1 and IE3 ATCGTC motifs have been shown to be strong Vmw175 binding sites (Faber and Wilcox, 1986b, 1988; Kristie and Roizman, 1986a,b; Muller, 1987; Roberts et al., 1988) whilst the IE2 promoter may only possess weak Vmw175 binding sites and the IE4/5 promoter is not detectably bound (Kristie and Roizman, 1986a; Faber and Wilcox, 1988).

The high affinity Vmw175 binding site at the transcriptional start site of IE gene 3 has therefore been implicated in the autorepression of this gene by Vmw175, and mutational studies of Vmw175 have revealed that the same domain of the protein is required to bind to this site and to repress transcription (DeLuca and Schaffer 1988; this thesis). An attractive but perhaps oversimplistic model for repression would be that the Vmw175 binding site acts as a classical prokaryotic operator site and that protein binding to this site prevents transcriptional initiation, either by stalling the initiation complex or by preventing its formation. The IE1 ATCGTC motif bound by Vmw175 is located between a CCAAT box motif and a potential Sp1 binding site, therefore binding of Vmw175 to this site might interfere with the normal activation of this promoter by these two sequence elements and their cognate factors (Faber and Wilcox, 1988). The IE4/5 promoter has been shown to be repressed by the addition of partially purified Vmw175 in vitro (Pizer et al., 1986).

Intriguingly the only other HSV promoter containing a perfect match to the ATCGTC motif is that of the gene encoding gD, which is regulated as an early gene (Cohen et al., 1980; Ikura et al., 1983; Johnson et al., 1986). This sequence, located at bases -101 to -106, has been shown to

be tightly bound by Vmw175 (Faber and Wilcox, 1986b; Tedder and Pizer, 1988) but lies outwith the minimal promoter responsive to Vmw175 transactivation (Everett, 1983, 1984a,b). This promoter has been shown to be moderately activated by partially purified Vmw175 *in vitro* (Beard *et al.*, 1986; Pizer *et al.*, 1986; Tedder and Pizer, 1988) but there has been a report that Vmw175 negatively regulates the gD promoter in biochemically transformed cells (Arsenakis *et al.*, 1988).

2.3 A detailed mutational analysis of the structure and function of Vmw110

Parallel to the mutational analysis of the structure and function of Vmw175 presented in this thesis, a complementary analysis of Vmw110 was performed in this laboratory. The predicted Vmw110 polypeptide contains 775 amino acids, and two introns are spliced out of the coding region of the primary transcript to form the mature mRNA (Perry *et al.*, 1986). Like IE gene 3, the sequence of IE gene 1 is G+C rich (75.4%) and encodes a protein with correspondingly unusual amino acid composition, biased towards residues with G+C rich codons.

One particular region of the protein was noted to have an interesting potential secondary structure; a region from residues 99-156 contains 9 cysteine (C) residues, including three C-n-n-C pairs which are thought to form a structural motif involved in the coordination of metal ions, a metal finger binding domain (see Figure 6; Berg, 1986). Such structures are often involved in nucleic acid binding but also in other functions such as protein:protein interactions (Frankel and Pabo; 1988). Gene 61 of VZV encodes a weakly homologous protein to HSV-1 Vmw110, with the conservation limited to the cysteine-rich region and all three of the C-n-n-C pairs conserved (Perry *et al.*, 1986; Davison and Scott, 1986).

Like Vmw175, Vmw110 contains a serine-rich region from residues 554-594, this region could potentially form a major site for phosphorylation of the protein.

A panel of plasmid-borne, in-frame insertion and deletion mutants of the gene encoding Vmw110 hve been

Figure 6: The potential "metal finger" binding domain of Vmw110

(a) $Cys\text{-}X_{2-4}\text{-}Cys\text{-}X_{2-15}\text{-}Cys/His\text{-}X_{2-4}\text{-}Cys/His$

(b)	Cys	$-X_2-$	Cys	$-X_6-$	His	$-X_2-$	Cys
	115		118		126		129
(c)	Cys	$-X_4-$	Cys	$-X_4-$	Cys	$-X_2-$	Cys
	129		134		139		142
(d)	Cys	$-X_2-$	Cys	$-X_{10}-$	Cys	$-X_2-$	Cys
	139		142		153		156

Figure 6: The potential "metal finger binding domain of Vmw110. (a) Consensus sequence of Berg (1986). (b) to (d) Three partially overlapping sequences in the second exon region of Vmw110 which fit the Berg consensus. The amino acid residue numbers are shown. (Taken from Everett, 1988a).

constructed and analyzed in transient transfection assays (Everett, 1987b, 1988a). Some of the more interesting of these mutants have been reintroduced into the HSV-1 chromosome in the native IE1 loci and their function has been analyzed in the infected cell (Everett, 1989).

The results using the plasmid-borne mutants revealed that the structural integrity of at least five regions of Vmw110 are important for the transcriptional transactivation of the gD promoter in synergy with Vmw175, but that the regions important for activation in the absence of Vmw175 were somewhat different (see Figure 7). This implies that the mechanism for these two activities is different. The cysteine-rich potential metal finger binding domain was essential for Vmw110 activity in the absence of Vmw175, but contributes less critically to activity in the presence of Vmw175. In contrast the carboxy-terminal region (5) is essential for synergistic function, but less important for independent action. This could indicate that this region is involved in interacting directly or indirectly with Vmw175, if any such physical interaction occurs.

The cellular location of the Vmw110 polypeptides in transfected cells was also affected by some deletions in the coding sequence. Cells transfected with wild-type Vmw110 exhibited a punctate pattern of nuclear immunofluorescence when stained indirectly with an antisera raised versus the carboxy-terminal of Vmw110. Several deletions spanning residues 474-509 were shown to be required for nuclear localization. This region contains a highly basic sequence RPRKRR similar to previously identified nuclear localization signals (Kalderon et al., 1984; Krippl et al., 1985). This sequence may not be sufficient for nuclear localization as a mutant with a large deletion spanning residues 509-638 was also cytoplasmically located (Everett, 1988a). Furthermore, mutant Vmw110 deleted from residues 475-548 is able to localize in the nucleus of cells infected with recombinant viruses expressing this polypeptide, perhaps in association with other IE products. This phenomenon could not, however, be reproduced in transfected cells (Everett, 1988a). Intriguingly several deletions in the carboxy terminal 125 amino acids of Vmw110 caused the polypeptide to give an

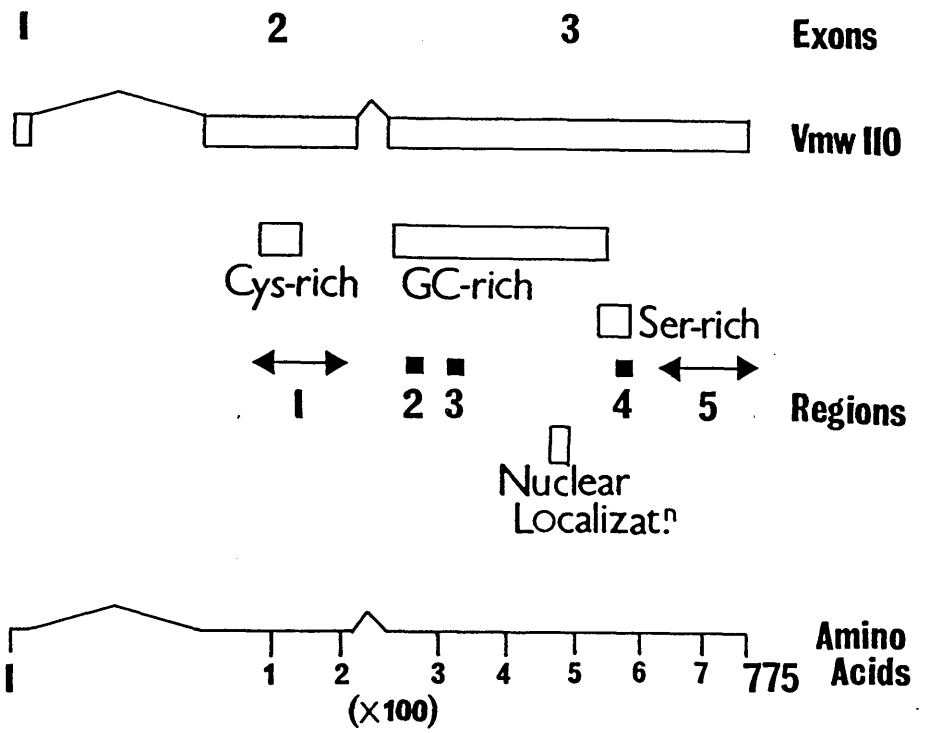


Figure 7: Structural and functional regions of Vmw110. The coding sequence of Vmw110 is composed of three exons, and encodes a protein of 775 amino acids. Regions of the protein particularly rich in cysteine and serine residues are highlighted, as is a region of the coding sequence markedly rich in GC residues. The limits of a sequence required for nuclear localization of the protein, at least in transfected cells, is shown. Five regions (1-5) of the protein which are sensitive to disruption of the transactivating activity of this protein in synergy with Vmw175 are shown.

abnormal pattern of diffuse nuclear staining in transfected cells, unlike the punctate nuclear staining seen with wild-type Vmw110. Mutations in this region may therefore cause a failure of Vmw110 to self-aggregate or localize in discrete sites of the nucleus (Everett, 1988a).

The growth properties of ten recombinant HSV-1 viruses encoding representative insertion and deletion mutants of Vmw110 have been examined (Everett, 1989). Deletions in the cysteine-rich region (1) had the most severe effects on virus growth, and were as impaired as deletion of the entire gene. Therefore this region, which is essential for the independent activation of gene expression by Vmw110, is the most important for the biological role of Vmw110 in the lytic cycle. The carboxy-terminal domain seemed less important in most cell types.

Although the viruses showed large differences in plaquing efficiencies and in one step growth curves, the mutations in Vmw110 had no effect on the yield of virus particles. The phenotypes of the mutants were dependent upon the growth state and type of cells used, but the patterns and amounts of viral polypeptide synthesis during high m.o.i. infections were similar in all cell types for the mutant and wild-type viruses. Only at low m.o.i. in the least permissive cell-types did the mutations in Vmw110 cause a reduction in viral gene expression. The defect was clearly shown to be at the level of gene expression when small numbers of particles were used to infect the less permissive cell-types. Furthermore, the ability of the viruses to activate a transfected early promoter (gD) was impaired in the mutant viruses. This was not cell-type dependent, however, and therefore did not simply reflect the varying efficiencies of gene activation by Vmw175 in the absence of Vmw110 (R.Everett, per.comm.).

Although the role of Vmw110 in the viral infection is now less obscure as a result of these comprehensive mutational studies, the exact mechanism of Vmw110 action is still a mystery, particularly its function in the absence of Vmw175 and the role of the cysteine-rich region. Clearly, like Vmw65, Vmw110 is important in the initial stages of infection, in determining the fate of the infecting virus

particle and in committing the virus to the lytic cycle. Whether this function is dependent upon the action of Vmw110 on early promoters in conjunction with Vmw175 or upon the promoter of the Vmw175 gene is unknown. The importance of the cysteine-rich region of the polypeptide implies that it is the (as yet unidentified) activity of this region which is critical in this process.

2.4 Mutational analyses of Vmw175

The main aim of the work presented in this thesis was to investigate the functionally important regions of Vmw175 by performing a detailed mutational analysis of the protein. It is therefore relevant to review previous studies which have contributed to our knowledge of the structure of Vmw175. Properties of the IE gene 3 DNA sequence, the predicted amino acid sequence and homology studies of the protein sequence have already been described above (Section 1D1.5).

Clues as to the functionally important regions of Vmw175 have been provided by fine structure mapping of a number of ts mutations within the coding sequence. Dixon and Schaffer (1980) have mapped the order and position of 9 ts mutations within the Vmw175 coding sequence using marker rescue and two-factor crosses. All nine mutations exhibited similar but not identical phentypes at the NPT. Two mutants, tsB28 and tsB32, which mapped towards the carboxy-terminus of the coding sequence were found to be more permissive for late gene expression than others. Preston (1981) has also mapped four ts lesions by similar procedures and found that the least permissive (leaky) mutant, tsK, maps nearer to the amino terminus of the protein. The DNA change responsible for the phenotype of tsK has since been determined at the nucleotide level and found to cause a single amino acid substitution (alanine to valine) at residue 475 (Davison et al., 1984). This mutation lies within a region of Vmw175 strongly conserved between Vmw175 and VZV140K (McGeoch et al., 1986a; see Section 1D1.5).

DeLuca et al., (1984) isolated two ts-Vmw175 HSV-1 mutants, ts48 and ts303, which overexpressed IE proteins at the NPT but synthsized a number of early gene products not

usually seen with other ts-Vmw175 mutants at NPT. These mutants were also capable of inducing 15-50% wild-type levels of viral DNA synthesis at NPT, although late gene products were under synthesized. These ts mutations therefore seemed to cause a block in gene regulation at late times of infection, but were able to transactivate a plasmid-borne early promoter (that of the tk gene) by superinfection of transfected cells. The DNA lesions of these two mutants were mapped to the same carboxy-terminal region of the coding sequence as the lesions in tsB28 and tsB32, which were also permissive for some early gene expression (Dixon and Schaffer, 1980; DeLuca et al., 1984). However, tsB32-Vmw175 did not seem to activate the cloned tk (early) promoter by superinfection or when subcloned onto a plasmid (DeLuca et al., 1984; DeLuca and Schaffer, 1985). Interestingly, the majority of ts-Vmw175 mutations seem to map to the more carboxy-terminal regions of the protein (region 4) and few have a phenotype as tightly restricted for viral gene expression as the more amino-terminal tsK lesion (in region 2) (Dixon and Schaffer, 1980; Deluca et al., 1984; Preston, 1981; McGeoch et al., 1986).

Viruses containing spontaneous (silent) and constructed (conditionally-lethal) deletions within the IE3 coding sequences have been isolated. Schrøder et al., (1985) described the characterization of a virus with a spontaneous deletion removing amino acids 209-236 of Vmw175 which was viable on non-complementing cells, although the plaques were atypically small and syncitial in morphology (syn^-). The viruses were also somewhat ts in plaquing efficiency and markedly so for DNA replication. This deletion removes 28 amino acids almost immediately adjacent to the serine and acidic residue-rich region of Vmw175 (176-206) which is conserved in HSV, PrV and VZV (see Sections 1D1.5 and 4.3.1). However, when attempts were made to rescue this virus with wild-type DNA fragments, viruses with wild-type plaque morphology and growth properties could be recovered which still possessed the 84bp deletion. This short section of Vmw175 is therefore non-essential for growth in tissue culture, and may not have detectable phenotypic effects. The phenotype of this spontaneous deletion mutant is very

similar to the phenotype of the virus deletion I15HBC reported in this thesis (Section 3F) which is deleted for Vmw175 residues 162-229.

The creation of cell lines which constitutively express Vmw175 has allowed the construction of recombinant HSV-1 and HSV-2 viruses with conditionally-lethal mutations in IE gene 3 (DeLuca et al., 1985; Russell et al., 1987b; Smith and Schaffer, 1987a; DeLuca and Schaffer, 1988). Initially these were used to construct viruses with gross deletions in Vmw175 which were useful as null mutants in the characterization of Vmw175 function and as parents for the substitution of alleles of IE gene 3 carrying more subtle changes in the coding sequence (DeLuca et al., 1985; DeLuca and Schaffer, 1987, 1988; this thesis).

The most rapid approach to investigating the structure and function of Vmw175 has been to create insertion and deletion mutants within the Vmw175 coding sequence and assay their activity in short term transfection assays (Mavromara-Nazos et al., 1986; DeLuca and Schaffer, 1987, 1988; Hubenthal-Voss et al., 1988; Paterson and Everett, 1988a,b; this thesis). An early intriguing observation, which suggested that much of the Vmw175 protein sequence was inessential for at least some of its activity, was that a plasmid encoding a mutant Vmw175 polypeptide truncated at residue 825 was capable of directing some (albeit reduced) transactivation of early and late promoters (Mavromara-Nazos et al., 1986).

A much more detailed analysis of the activity of truncated mutants of Vmw175 has been conducted by DeLuca and Schaffer (1987) who constructed a panel of Vmw175 mutants by inserting oligonucleotides encoding translational stop codons in all three reading frames into various positions of the cloned IE gene 3. By assaying the activity of these mutants in transfection assays they showed that the amino terminal 60% of Vmw175 was sufficient to direct transactivation of the (early) HSV-1 tk promoter and autorepression of the IE3 promoter linked to the CAT gene. Furthermore, the amino-terminal 90 amino acids of the protein proved to be innessential both in transient assays and when this mutation was recombined back into virus.

Insertion of a stop codon within the first 90 codons seemed to permit translational initiation at the second methionine codon, located at amino acid 90, and direct synthesis of a polypeptide 10kDa smaller than wild-type (DeLuca and Schaffer, 1987). The ability of the truncated mutants to complement growth of a virus with a large deletion within IE gene 3 was also assayed. All, apart from the 90 residue amino-terminal deletion, were severely impaired. This indicated that the carboxy-terminal 40% of Vmw175 may be directing functions not revealed by the transient assays (see results Section 3C3).

An interesting phenomenon observed in both the transactivation and complementation assays was that mutants expressing 60% of the protein had greater activity than ones expressing 80%. This suggests that a domain of the protein between these sites inhibits or negatively regulates Vmw175 function, either directly, or as a consequence of abnormal protein conformation.

Several of these mutated genes were recombined back into the HSV genome in their correct chromosomal loci, and the growth properties of the recombinant viruses investigated (DeLuca and Schaffer, 1988). The properties of the truncated and deleted Vmw175 polypeptides produced by these recombinant viruses are shown in Table 3. The mutants were found to belong to one of three classes with respect to their growth properties and gene regulation:

I: Identical to wild-type with respect to gene expression and replication; only recombinant n6 (the amino terminal 90 amino acid deletion, see Table I) showed this phenotype.

II: Mutants express only IE gene products; derivatives of Vmw175 with deletions extending into the first 60% of the coding sequence possess this phenotype.

III: Mutants able to induce some early protein synthesis but not to wild-type levels. IE3 mRNA and Vmw175 protein overaccumulates although repression at the level of transcription, as measured by transcription run-on assays in isolated nuclei, is not seen. Deletion mutants expressing derivatives of Vmw175 with the first

Table 3: Properties of recombinant viruses expressing truncated or deleted Vmw175 polypeptides (DeLuca and Schaffer, 1988).

1 Virus	2 Truncation/ Deletion	3 Comments	4 Class	5 Localiz^{n}	6 DNA Binding
n6	11	Restarts at 90	I	-	+
n12	251		II	c	-
n214	590		II	c	(+)
n208	773		III	n	+
n18	818		III	n	+
n215	1028		III	n	+
d120	171	(Deletion)	II	?	-
d202	680	(Out of frame deletion)	II	?	-
d156	680-857	(In frame deletion)	II	c	-
d2	185-309	(In frame deletion)	II	n	-

Table 3: Properties of recombinant viruses expressing truncated or deleted Vmw175 polypeptides (DeLuca and Schaffer, 1988). Column 2 records the position of the inserted translational stop codon or the deletion end-points of the mutated Vmw175 polypeptides produced by the recombinant viruses (column 1). These mutations are further described in column 3. The phenotypic class (as described in the text) of each mutant is given in column 4. Column 5 describes the cellular localization of the mutant polypeptides expressed in the infected cell (n: nuclear; c: cytoplasmic) and column 6 records the ability of the mutant proteins to bind to the IE3 cap site (n214 only binds at elevated salt concentrations, see text).

60% of the coding sequence uninterupted possess this phenotype.

None of the class II or III mutants were able to grow on non-complementing cell lines. The nuclear localization signal of Vmw175 seemed to be located between the ends of the n214 and n208 polypeptides (residues 590-773), however, all of the mutant forms were impaired with respect to subnuclear localization and failed to localize to discrete areas of the nucleus as seen with wild-type or n6-Vmw175.

All of the polypeptides with the exception of d120 were phosphorylated at near normal levels, implicating the amino acids between the end points of d120 and n12 (171-251) in phosphorylation. This would support the involvement of the serine-rich tract (186-204) in phosphorylation.

DeLuca and Schaffer (1988) also examined the ability of the mutant polypeptides to bind to the ATCGTC consensus binding site at the IE3 start site. Viral specific complexes, containing Vmw175, were seen with all of the class I and III mutants capable of autoregulation of IE3 transcription. Under the conditions of binding used in these assays the Vmw175 DNA complexes were salt-labile when the concentration of NaCl was raised from 15 to 65mM; this contrasts with the results presented in tnis thesis and by others (Faber and Wilcox, 1988) that show that DNA binding is stable at much higher salt concentrations. However, at these elevated salt concentrations some of their mutants were able to form novel complexes of greater mobility in gel retardation assays, including a class II mutant truncated at amino acid 590. It was suggested that this complex may represent monomer binding , whilst the larger, more salt-labile complex represents multimers of Vmw175 bound to DNA.

DeLuca and Schaffer (1988) presented a map of the physical and functional domains of Vmw175 based on their own and earlier studies. This map is reproduced in Figure 8. The results and conclusions presented in this thesis are in good agreement with their results (see Chapters 3 and 5).

It is also relevant here to briefly consider some attempts that have been made to accurately map the Vmw175

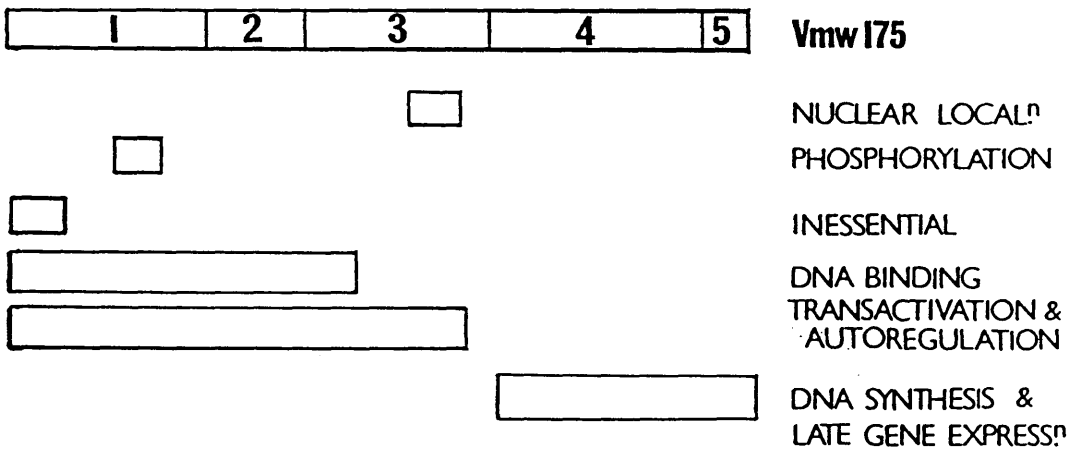


Figure 8: Functional domains of Vmw175 (after DeLuca and Schaffer, 1988). The Vmw175 sequence is divided into the five structural domains proposed by McGeoch et al., (1986) (see Figure 5). The extent of the functionally important regions of the molecule as suggested by DeLuca and Schaffer, (1988) are shown beneath the protein sequence.

DNA binding domain by a variety of approaches. Kristie and Roizman (1986b) have reported that binding of monoclonal antibody H950 to Vmw175 abolishes DNA-binding to the IE1 promoter. The epitope for this antibody has been mapped to within amino acids 21-36 (Hubenthal-Voss et al., 1988) and therefore lies within a section of region 1 shown not to be required for DNA binding by the results of DeLuca and Schaffer (1988) and the results presented in this thesis (Paterson and Everett, 1988b; Section 3D). Therefore it is likely that the antibody is blocking DNA binding through longer range steric affects.

Hubenthal-Voss et al., (1987) have also attempted to map the DNA binding domain of Vmw175 by using short synthetic oligopeptides to interfere or compete with functional protein domains involved in binding to the DNA recognition sites in the IE1 promoter. Their results, which mapped regions important for DNA binding activity near to the amino terminus, are at variance with the results presented in this thesis (Paterson and Everett, 1988b; Section 3D). Indeed it has been shown that the region they investigated seems to be of little importance, at least in binding the IE3 recognition site (Paterson and Everett, 1988b; this thesis). Hubenthal-Voss et al., (1988) showed that Vmw175 in transfected nuclear extracts could bind to DNA and that Vmw175 truncated at residue 825, but not residue 519, could also bind to DNA. From this result they suggested that a DNA binding domain lay between residues 519 and 825, a hypothesis not borne out by results presented in this thesis (Paterson and Everett, 1988b). Furthermore, it is not clear how the interpretation of their results using nuclear extracts to assay Vmw175 DNA binding is affected by the mapping of a nuclear localization signal to within this region (DeLuca and Schaffer, 1988; Paterson and Everett, 1988a).

SECTION 1E: The mechanism and regulation of transcriptional initiation by RNA polymerase II.

1. RNA polymerase II and factors required for initiation of transcription of class II promoters.

Regulation of the initiation of gene transcription is the fundamental level at which eukaryotic gene expression is controlled. In this section of the thesis I shall attempt to review transcription from inducible class II promoters.

Three separate enzymes have been defined which transcribe DNA to form RNA in the nucleus of eukaryotic cells. These eukaryotic RNA polymerases are large, multisubunit complexes composed of 9-14 polypeptides. The RNA polymerases I and III have specialized roles in cellular gene expression and transcribe a small subset of the genomic DNA, recognizing well defined promoter elements. RNA polymerase II transcribes a much greater portion of genomic DNA sequences and responds to a greater diversity of gene expression signals.

The two largest subunits of RNA polymerase II share structural homology with the two largest subunits of prokaryotic RNA polymerases. However, the carboxy-terminal domain of the largest subunit is unique in that it is composed of a long tandem repeat of a heptapeptide consensus sequence [YSPTSPS]. The exact number of repeat units varies both within and between species, from 27 to 52 (in yeast and mouse respectively) (Corden et al., 1985; Allison et al., 1985; Nonet et al., 1987). Studies in yeast have shown that the length of this tandem repeat element is functionally redundant, and yeast strains encoding polymerases with 10-13 repeats are viable (Nonet et al., 1987). In Drosophila a longer repeat domain appears to be essential (Zehring et al., 1988).

This repeat unit appears to form a well defined secondary structure rich in hydrophilic (hydroxyl) side chains, which may project out from the globular RNA polymerase holoenzyme. In a minimal in vitro system this structure is non-essential for RNA synthesis and can be removed by proteolysis without disrupting the accurate initiation of transcription. This domain may therefore be

involved in the regulation of transcription (Zehring et al., 1988). It has been suggested that the exact size and sequence of this carboxy-terminal regulatory domain is not critically important, but that the hydroxyl groups might be involved in interacting in a fairly non-specific manner with transcription activating proteins. In particular it has been suggested that the acidic activating regions found on many transactivating proteins (Section 1E3.3) would be able to form multiple non-specific carboxylate-hydroxyl contacts with this structure (Sigler, 1988). These carboxyl groups might also be the target for phosphorylation by protein kinases, perhaps catalyzed by the acidic residues of the transcriptional activators.

In addition to purified RNA polymerase II holoenzyme a number of ancillary factors have been defined in vitro which are essential for the accurate initiation of transcription from templates encoding a class II promoter which include a TATA box element (see Section 1E2.1). Three partially purified transcription factors TFIIB, D and E are required in addition to RNA polymerase II for the transcription of such a template in vitro (Matsui et al., 1980; Sawadogo and Roeder, 1985a; Moncollin et al., 1986).

Kinetic studies have defined several stages in the process of transcriptional initiation. Commitment to transcription involves recognition of the promoter by TFIID which binds to the TATA box element to form a stable complex in the absence of other transcription factors and RNA polymerase (Davison et al., 1983; Sawadogo and Roeder, 1985b; Moncollin et al., 1986; Zheng et al., 1987). Recognition of the promoter by TFIID allows the association of RNA polymerase II to form an intermediate, stable preinitiation complex, which is converted to an activated complex by the addition of TFIIB and E. DNA binding studies have shown that protein/protein and protein/DNA interactions are important in the formation of these complexes, and that the extent of DNA sequences bound by TFIID are extended by TFIIB and E (Van Dyke et al., 1988). An energy dependent step, involving the hydrolysis of ATP, then alters the conformation of the activated complex, perhaps involving the loss of TFIIB and E, and allows initiation of transcription

upon the addition of rNTPs (Fire et al., 1984; Van Dyke et al., 1988).

Binding of TFIID to the TATA box appears to be the critical step in the formation of the preinitiation complex and is certainly important in determining the rate at which transcription is initiated. It is therefore not suprizing that recent studies have shown that this factor seems to be the target for activation by both viral and cellular transcriptional activator proteins (Section 1E3.5).

2. DNA sequences involved in the control of RNA polymerase II initiation.

The genes transcribed by RNA polymerase II can be divided into two subclasses on the basis of their expression and the promoter sequences controlling their transcription. Genes encoding so called "housekeeping" products, common to all cell-types, are transcribed at low levels in most cells and have poorly defined promoters devoid of obvious recognition elements. The majority of class II promoters, however, share a basic structure which generally includes a TATA box element and various upstream regulatory elements. These promoters are responsive to various forms of regulation, determined both by tissue type and developmental stage, and in many cases respond to transient forms of induction.

The DNA sequences required for the accurate and efficient initiation of transcription are generally located within 100 bases upstream of the transcriptional start site (+1). The efficiency of the basal level of transcription from a promoter is determined by its constitutive promoter elements.

2.1 The TATA box.

The minimal promoter includes an AT rich region designated the TATA box located at around -25 to -30 bases upstream from the start site. This forms an essential component of the transcriptional machinery and is essential for accurate initiation of transcription in vitro (reviewed Maniatis et al., 1987; Nakajima et al., 1988). The TATA box is bound by a factor, or family of factors, called TFIID

(Davison et al., 1983; Sawadogo and Roeder, 1985b; Moncollin et al., 1986). TFIID has been partially purified and shown to interact with a variety of class II promoters and is necessary for their transcription in vitro. However, there may be multiple forms of this TATA box binding factor, as TFIID was found to interact differently with distinct promoters (Nakajima et al., 1988). There is also evidence that there is actually a family of TATA box sequences, which react differently to viral transactivation and therefore may be bound by different members of a TFIID family (Simon et al., 1988; Everett, 1988b). In addition the TATA box has been implicated in mediating the response to viral IE transactivators in vivo and in vitro (Coen et al., 1988; Simon et al., 1988; Wu et al., 1987; Wu and Berk, 1988; Abmayr et al., 1985, 1988).

2.2 Upstream promoter elements.

Promoters contain one or more upstream promoter elements (UPEs) usually positioned within 100 bases upstream from the TATA box sequence (reviewed by Maniatis et al., 1987). These sequences are modular in nature, consisting of arrays of short (10-12bp) recognition elements which interact with specific cellular DNA binding proteins (transcription factors). Several of these UPE sequences are common to a wide variety of promoters and are bound by factors which are found in many cell-types (for example CCAAT, bound by CP1, CP2 or NFI; GGCGGG, bound by Sp1). Other elements are found less frequently and may have a role in determining cell-type specificity. UPEs have also been characterized which confer inducibility on a promoter and may be considered as inducible enhancer elements. The response elements to heat shock (HRE), heavy metals (MRE) and steroid hormones are all examples of this type of regulatory motif, which are only bound by their cognate transcription factor in response to an intracellular signal.

Although the orientation relative to and distance from the TATA box of UPEs does not in general appear to be critical for their function, in some instances the spacing between elements has been observed to be important, especially when these elements are closely juxtaposed. For

example the introduction of half turns in the DNA helix between UPEs in the SV40 early promoter interferes with transcription from the normal start site. This provides evidence that the activity of UPEs is mediated through the proteins bound at the site, not the DNA sequence itself (Takahashi et al., 1986). It has also been suggested that at least one of the upstream regulatory elements must lie close to the TATA box, to mediate interaction with the TATA box binding factor TFIID (Lin et al., 1988).

2.3 Enhancer elements

Enhancers are DNA sequence elements which can activate transcription from a cis-linked promoter in an orientation and distance independent manner. Like promoters, enhancer elements are also composed of multiple DNA sequence elements bound by ubiquitous or cell-type specific transcription factors, and indeed the mechanisms by which these sequences and their cognate transcription factors activate transcription may be indistinguishable. Furthermore, at least some UPEs can function as components of enhancers as well as of promoters. One such element is the octamer binding site which is found in a variety of promoters and immunoglobulin enhancers (Bohmann et al., 1987a; Parslow et al., 1987). The functional distinction between enhancers and UPEs (that is the "action at a distance" property of enhancers) probably reflects the arrangement and number of transcription factor recognition elements in enhancers, rather than the mechanism by which they act.

The structure and function of the enhancer encoded by the papovavirus SV40 has been dissected in detail. This enhancer is composed of multiple sequence motifs that stimulate transcription synergistically and are organized at a higher level into two or three functional domains which are active when multimerized (Zenke et al., 1986). These motifs are bound by a variety of ubiquitous and cell-type specific factors, which probably accounts for the wide "host-range" of this enhancer. Binding of these factors to their cognate sites is strongly correlated to their trancriptional activity (Davidson et al., 1986; Wildeman et al., 1986; Nomiyama et al., 1987). Unlike the inducible

motifs bound by the steroid hormone receptors, the individual SV40 enhancer motifs have no independent activity, but enhance transcription only when oligomerized in association with themselves or a second motif to form a multi domain enhancer (Fromental et al., 1988; Davidson et al., 1988).

3. Transcriptional activation.

3.1 Factors interacting with upstream promoter elements and enhancers.

Cellular proteins ("transcription factors") capable of binding to 10-12bp DNA motifs in the upstream regulatory regions of promoters and enhancers have been identified by a variety of in vitro DNA binding assays. These proteins are generally inferred to mediate transcriptional regulation, and in many cases a direct correlation between DNA binding and transcriptional activation has been demonstrated both in vivo and in vitro (reviewed by McKnight and Tjian, 1986; Maniatis et al., 1987; Jones et al., 1988; Ptashne, 1988; Wingender, 1988).

(i) Spl

The first cellular transcription factor to be characterized in detail was Spl, which binds to GC box motifs (GGCGGG) in various promoters and activates this subset of genes in mammalian cells (reviewed Kadonoga et al., 1986; McKnight and Tjian, 1986). Spl has been purified by DNA affinity chromatography and shown to bind to GC boxes in vitro and stimulate transcription (Briggs et al., 1986). A cDNA for the transcript encoding Spl has been cloned and shown to encode a protein with a carboxy-terminal DNA binding domain. This region has three continuous zinc finger motifs which seem to be involved in DNA binding (Kadonoga et al., 1987). Two regions of the protein identified as being involved in mediating transcriptional activation are distinguished by containing an unusually high proportion of glutamine residues (Courey and Tjian, 1988).

Recently it has been shown that Spl bears multiple O-linked N-acetylglucosamine (GlcNAc) monosaccharide residues and that this post-translational modification may

contribute towards the transactivating activity of Sp1 (Jackson and Tjian, 1988). A large number of other cellular class II transcription factors were also demonstrated to be GlcNAc glycosylated, including Ap1, Ap2, and CCAAT binding factor, raising the interesting possibility that this is an important conserved modification of class II transcription factors.

(ii) CCAAT box binding factors CP1, CP2 and NFI.

The CCAAT box sequence motif is found in the upstream regulatory regions of many class II promoter and has been demonstrated to be important for promoter function (Jones et al., 1985; Graves et al., 1986; McKnight and Tjian, 1986). It has been shown that there is a family of at least three multisubunit CCAAT-binding proteins composed of heterologous subunits, and that these bind to distinct CCAAT box recognition sequences which vary in their flanking DNA sequences. DNA binding is correlated with potentiation of transcription (and in one case with viral replication) (Chodosh et al., 1988a).

The protein CP1 is composed of two heterologous subunits which are conserved between yeast and human cells. The yeast and human proteins have identical specificities and the subunits are functionally interchangeable (Chodosh et al., 1988b). Recognition of the CCAAT box motifs is therefore more complicated than binding of Sp1 to the GC box motif, with a variety of different proteins recognizing variants on a common core element. It remains to be seen how the different CCAAT binding proteins are related.

Studies on the regulation of the human thymidine kinase gene have revealed variations in the nature of the complexes with the CCAAT box, which are implicated in the cell-cycle dependent control of transcription (Knight et al., 1987).

(iii) Ap1 and c-Fos

The transcription factor Ap1 interacts with the TPA-inducible promoter and enhancer element TGACTCA (Lee et al., 1987a,b) and is encoded by the cellular proto-oncogene c-jun (Bohmann et al., 1987b; Angel et al., 1988; Bos et al., 1988). The tumour-promoting agent TPA stimulates Ap1

DNA binding activity by a post-translational mechanism, which may involve the TPA mediated stimulation of protein kinase C (Angel et al., 1987). The product of a second cellular proto-oncogene c-Fos has been shown to interact with Apl and to cooperate in the stimulation of transcription of Apl responsive genes (Sassone-Corsi et al., 1988; Rauscher et al., 1988; Chiu et al., 1988). Furthermore c-Fos can function as a transcriptional activator in yeast when fused to a site specific binding domain (Lech et al., 1988), perhaps indicating that c-Fos requires Apl to localize at responsive promoters in much the same way as Vmw65 interacts with the octamer binding protein to activate transcription from cis-linked promoters (Section 1C2). Apl and c-Fos interact together to form a heterodimer which has greater affinity for DNA than Apl homodimers; c-Fos homodimers do not bind to DNA (Nakabeppu et al., 1988; Halazonetis et al., 1988). The DNA binding specificity of Apl is identical to that of the yeast transcriptional activator GCN4 and the amino acid sequence of their DNA binding domains is conserved; indeed the DNA binding domain of Apl can substitute for that of GCN4 (Struhl, 1987). Clearly, like the CCAAT binding protein CP1, this transcriptional regulatory system has been conserved in evolution, which has important implications not only in the study of gene regulation but also of oncogenesis. The cAMP response eleme nt (CRE) is similar in sequence to the Apl binding site motif, but is bound by a protein (ATF or CREB) unrelated to Fos or Jun (reviewed, Curran and Franza, 1988).

(iv) ATF and TFIID interactions.

ATF is a mammalian transcription factor which binds to sequences found in many adenovirus and other promoters (Jones et al., 1988). Studies on the in vitro activity of the purified ATF have suggested a mechanism by which transcription factors binding to upstream sequences may activate transcription. Purified ATF can bind to upstream promoter elements in the adenovirus E4 promoter to potentiate transcription. In vitro DNA binding assays have revealed cooperative interactions between ATF and TFIID binding to this promoter. ATF binding does not increase the

rate of TFIID binding but appears to stabilize the preinitiation complex and facilitate RNA polymerase and TFIIB and E promoter recognition. The cooperative interactions between ATF and TFIID extend the DNA sequences bound by both proteins. The role for ATF appears to be transient, and dissociation of ATF from the complete initiation complex does not destabilize the initiation complex (Horikoshi et al., 1988b; Hai et al., 1988a).

(v) Factors interacting with enhancer elements.

The transcription factors that interact with recognition elements within enhancers are thought to be functionally equivalent to those binding to upstream promoter sites. This has been demonstrated experimentally by cloning the recognition element for NF-$_k$B from an immunoglobulin enhancer to a new position next to a TATA box to create a novel NF-$_k$B responsive promoter (Wirth and Baltimore, 1988).

Indeed there is overlap between the control elements found in promoters and enhancers. For example the binding sites for octamer binding protein OTF-1 (OCT-1) are found in a variety of promoters and enhancers (Rosales et al., 1987; Bohmann et al., 1987a) and overlap with the HSV Vmw65-responsive TAATGARAT elements (O'Hare and Goding, 1988; Gerster and Roeder, 1988). This same octamer element, however, can be bound by two or more proteins with different tissue distributions (Rosales et al., 1987). The ubiquitous octamer binding protein OTF-1 has been purified and binds to the histone H2B promoter in vitro to activate transcription (Fletcher et al., 1987) whilst the lymphoid specific factor OTF-2 (OCT-2) activates transcription of the immunoglobulin promoter in vitro (Scheidereit et al., 1987). The sequence of OTF-2 reveals that it encodes a homoeobox domain involved in DNA binding (Ko et al., 1988). OTF-1, OTF-2 and another transcription factor Pit-1 or GHF-1 share a conserved 160 amino acid element (POU) including the 60 amino acid homoeobox domain. This region is also conserved in the evolutionarily distant C.elegans developmental control protein Unc-86. The whole POU region appears to be involved in DNA binding (reviewed Marx, 1988; Robertson, 1988; Levine and Hoey, 1988).

In addition to exhibiting tissue specific distributions many transcription factors recognizing enhancer elements show further levels of control, and respond to variety of signal tranduction pathways. OTF-1 (like a CCAAT binding protein) has been reported to be active in a cell-cycle dependent manner (Fletcher et al., 1987). The activity of other enhancer binding proteins is modulated by treatment of the cells with TPA (Apl, Ap2) or in response to cAMP (Ap2) (Angel et al., 1987; Lee et al., 1987b; Imagawa et al., 1987). Heat shock induces the response to heat shock transcription factor (Parker and Topol, 1984) whilst hormone binding receptors only activate transcription when liganded to their cognate hormone (Section 1E3.2).

3.2 Hormone binding receptors.

Study of the steroid and thyroid hormone receptor transcription factors has been of particular interest because these receptors directly respond to intercellular messages to modulate cellular gene expression. Vertebrates encode a conserved superfamily of receptors for steroid and thyroid hormones and retinoic acid related ligands (reviewed by Evans, 1988; Green and Chambon, 1988). Each protein encodes a central DNA binding domain of about 70 amino acids which includes two conserved potential zinc binding fingers, the first of which determines the specificity of DNA binding to upstream promoter elements (Green and Chambon, 1987; Green et al., 1988). This short DNA binding domain may also encode the hormone inducible activation function of the steroid hormone receptors (Hollenberg et al., 1987, Kumar et al., 1987). A large carboxy-terminal domain, separated from the DNA binding domain by a hinge region, binds to the hormone ligand. The amino-terminal domain is not conserved in size or sequence between different receptors and does not seem to be very important for function but may contribute towards the efficiency of activation (Giguere et al., 1986, Kumar et al., 1987). Transcriptional activation by the hormone receptors is mediated through upstream promoter elements which are bound by specific receptors and function as inducible enhancers.

The activity of the receptor proteins is regulated by

hormone binding. Nuclear localization of the glucocorticoid steroid receptor (GR) requires hormone binding, and the unliganded GR may be complexed with the heat shock protein hsp90 in the cytoplasm (Picard and Yamamoto, 1987; Denis et al., 1988). Upon hormone binding the receptor can dissociate from hsp90 and translocate to the nucleus where its DNA binding activity is unmasked. Hormone liganding may also potentiate the dimerization of receptors (Kumar and Chambon, 1988). Although unliganded steroid receptors can bind to DNA in vitro, it appears that the unliganded steroid binding domain represses the transcriptional activation by the receptors in vivo by directly or indirectly interfering with DNA binding (Becker et al., 1986; Willmann and Beato, 1986; Denis et al., 1988). If the steroid binding domain is deleted this control is alleviated and the receptor becomes constitutively active (Hollenberg et al., 1987; Godowski et al., 1987; Picard et al., 1988). The unliganded steroid binding domain represses transcriptional activation and DNA binding whether positioned at the carboxyl or amino terminus, and when fused to Ela also regulates the activity of this transcriptional activator, possibly by associating with hsp90 (Picard et al., 1988).

The virally transduced oncogene v-erbA is homologous to the cellular thyroid T_3 hormone receptor c-erbA (Sap et al., 1986; Weinberger et al., 1986) but is truncated for the carboxy-terminal hormone binding domain and does not bind thyroid hormone (Munoz et al., 1988). The oncogenic activity of v-ErbA appears to depend on the DNA binding domain suggesting that its activity may be due to constitutive (non-hormone regulated) transcriptional regulation (Privalsky et al., 1988). v-ErbA has been shown to specifically repress the transcription of at least one gene (Zenke et al., 1988).

The hormone receptor proteins are capable of transcriptional repression, which seems to operate by a variety of different mechanisms. The T_3 receptor (T_3R, c-ErbA) recognizes a similar sequence motif to the oestrogen receptor (ER), but whilst T_3R can bind to the ERE (oestrogen responsive element) this binding does not result in activation, but inhibits oestrogen dependent activation

(Glass et al., 1988). Therefore T_3R would appear to repress this ERE by binding in an inactive form. However, steroid receptor mediated repression of the rat prolactin gene does not require the DNA binding domain. In this case repression is mediated through the binding site for a separate transcription factor Pitl, suggesting that inhibition can be mediated by protein/protein interactions between steroid receptors and other transcription factors, and does not require direct DNA binding (Adler et al., 1988).

Glucocorticoid response elements (GREs) have been demonstrated to mediate repression of transcription in vitro and appear to be bound by an unidentified cellular factor, suggesting that GREs may be negatively regulated in the absence of GR binding (Langer and Ostrowski, 1988).

Little is known about how steroid hormone receptors activate transcription other than that this activation is correlated with DNA binding. However, glucocorticoid induction of the MMTV promoter has been shown to involve receptor mediated establishment of a transcription factor complex, not activation of a pre-existing complex, (Cordingley et al., 1987). Furthermore, it has recently been shown that GR and a cellular transcription factor bind cooperatively to their adjacent GRE and CACCC box binding sites. This cooperativity depended upon the exact spacing between the binding sites, which was thought to indicate that cooperativity was achieved by protein/protein interactions (Schule et al., 1988a). The action of steroid hormone receptors may therefore, at least in some cases, involve or require participation of other transcription factors bound to the upstream regulatory region. Glucocorticoid and steroid responsive elements (GRE and ERE) have been shown to confer hormone inducibility on a minimal promoter when placed immediately upstream of the TATA box, suggesting that the receptor protein in conjunction with TFIID is able to form an active transcription complex. However, if the GRE is placed 350 bases upstream from the TATA box a second adjacent transcription factor binding site was required to confer hormone inducibility. Binding sites for Spl, NFl, CCAAT-binding protein, CACCC-binding protein

and the glucocorticoid receptor itself could modulate this activity, suggesting that GR can function in synergy with a large number of transcription factors (Strahle et al., 1988; Schule et al., 1988b).

Regions of the hormone receptors involved in activation and distinct from those involved in DNA binding have been defined. Mutational analysis has defined one region towards the amino terminus of the GR that is involved in the activation of transcription (Giguere et al., 1986). Although this region is acidic, like the activating regions of other transactivating proteins (Section 1E3.3), it is not conserved between different receptor proteins. A recent study has identified a carboxy-terminal 30-amino acid peptide of GR which also acts as an activating domain (Hollenberg and Evans, 1988). This peptide is also acidic but structurally unrelated to the amino-terminal region. Other studies indicate that multiple regions of the receptor proteins may be involved in activating transcription (Green and Chambon, 1988).

3.3 Transcriptional activators in yeast.

Recent studies have shown that transcriptional regulation in the yeast S.cerevisiae shares many properties with transcriptional regulation in higher eukaryotic cells. Indeed several mammalian transcription factors have been shown to have yeast homologues as discussed in the preceding sections. However, most attention has been focussed on two yeast transcription factors, GAL4 and GCN4, which bind to upstream activating sites (UASs) to activate transcription from an adjacent gene. Like mammalian enhancers these UASs are modular sequence elements which can be moved relative to the critical cis-element, the TATA box (reviewed by Guarente, 1988).

Both the GAL4 and GCN4 proteins have been demonstrated to consist of distinct domains specifying site-specific DNA binding (to the cognate UAS) and transcriptional activation from a cis-linked promoter (reviewed by Struhl, 1987). The DNA binding activities are specified by short domains at the amino and carboxyl termini of GAL4 and GCN4 respectively. The GAL4 DNA binding domain contains two potential zinc

fingers which might be involved in DNA binding (Hope and Struhl, 1986; Keegan et al., 1986). The DNA binding domain of either protein can be substituted by that of a heterologous E.coli protein, LexA, and the novel fusion proteins activate transcription from promoters linked to LexA binding sites (Brent and Ptashne, 1985; Hope and Struhl, 1986).

The DNA binding activity of the GAL4 and GCN4 proteins does not itself cause transcriptional activation. This is dependent upon distinct activating regions of the protein. Indeed DNA binding by mutant GAL4 polypeptides deficient in transactivation causes repression when the UAS_{GAL} is appropriately positioned in front of a gene (Keegan et al., 1986).

The activating regions of GAL4 and GCN4 have been defined as stretches of 60-100 amino acids bearing a net negative charge. These regions activate transcription when fused to a DNA binding domain (Hope and Struhl, 1986; Ma and Ptashne, 1987a). It seems to be the acidic character of these activating regions that is their most important feature, perhaps when presented on an amphipathic helix, and as such these activating regions are less precisely defined structures than DNA binding domains (Ma and Ptashne, 1987a; Gill and Ptashne, 1987; Hope et al., 1988). Indeed short acidic oligopeptides cloned at random from E.coli or synthesized chemically can act as efficient activating regions when fused to the GAL4 DNA binding domain (Ma and Ptashne, 1987c; Giniger and Ptashne, 1987). The 78 amino acid highly acidic tail of the HSV-1 transinducing factor Vmw65 has also been shown to function as an activating region when fused to the GAL4 DNA binding domain (Sadowski et al., 1988). This acid tail is essential for the transactivation activity of Vmw65 (Friedman et al., 1988; Ace and Preston, per.comm.) and presumably activates transcription in human cells by a similar mechanism to other acid activating regions (Section 1C2.2).

The ability of this type of structural motif to activate transcription is therefore not restricted to yeast, and furthermore the GAL4 protein will function in Drosophila and mammalian cells to activate transcription from promoters

linked to the UAS_{GAL} sequence (Webster et al., 1988; Kakidani and Ptashne, 1988; Fischer et al., 1988). Other activating regions from mammalian transcription factors will function when fused to the GAL4 binding domain, including the Ela activating region (*Lillie and Green, cited in Ptashne, 1988), and the GAL4 and GCN4 activating regions function when fused to the ER DNA binding domain (Webster et al., 1988).

Evidence is now emerging as to how these acidic activating regions may function to increase transcription. Over expression of these domains seems to cause a general suppression of transcription from promoters not responding to these factors, suggesting that the acid domain is competing for an essential component of the transcriptional apparatus, possibly TFIID or RNA polymerase itself (Gill and Ptashne, 1988; Ptashne, 1988). The acidic domain from GAL4 seems to be able to function synergistically with other mammalian transcription factors, when, for example, an UAS_{GAL} is positioned next to the SV40 enhancer or a GRE (Webster et al., 1988; Kakidani and Ptashne, 1988).

Recent reports suggest that at least in mammalian cells TFIID is the direct target for GAL4 mediated activation. Binding of GAL4 in vitro to a UAS_{GAL}-TATA promoter template qualitatively alters the TFIID/TATA box interactions to extend DNA binding beyond the region -15 to +1. Mutants of GAL4 lacking an acid activating region bind to DNA but fail to transactivate and do not induce these changes in TFIID binding (Horikoshi et al., 1988a). GAL4 protein has also been shown to function in synergy with the TATA box binding factor in vitro to stimulate transcription, providing that the UAS_{GAL} binding site is positioned adjacent to the TATA box. Alternatively, more remotely bound GAL4 can exert a synergistic effect through a second transcription factor (ATF or USF) bound adjacent to the TATA box (Lin et al., 1988). Both these studies indicated that GAL4 has a third cryptic acid activating domain, which effects TFIID binding and transactivation in vitro, but does not function in vivo.

3.4 Viral transactivators.

A common feature of many viruses is that they encode

transcriptional activator proteins which regulate the expression of viral genes. The effect of these proteins is sometimes specific to viral promoter sequences, but more often non-selective in the promoter sequences they transactivate. These gene products are generally encoded by immediate early or early genes and potentiate the expression of later temporal classes of genes. The adenovirus Ela protein is one of the best studied examples of a promiscuous transactivator protein.

(i) Structure of the Ela proteins.

The adenoviruses Ad2 and Ad5 encode a family of related proteins derived from alternative splicing of the Ela gene transcript. The major products are translated from 13S and 12S mRNAs and encode 289 and 243 residue polypeptides respectively. The larger protein is essential for adenovirus growth.

The Ela products are complex multifunctional proteins which can activate a variety of viral and cellular genes *in trans*, repress the effect on transcription of viral and cellular enhancers, induce cellular DNA synthesis and mitosis, and participate in transformation (in conjunction with adenovirus Elb products or the *ras* gene product).

Mutational studies have been performed in order to define the domains of the protein which are essential for each individual function and a functional map of the protein has been derived (Figure 9). Three critical functional domains have been defined, which are conserved between different adenovirus serotypes. Protein domains (PD) 1 and 2 are essential for the induction of DNA synthesis, transformation and the repression of enhancer function, activities that may not be essential for virus growth as PD1 is non-essential (Smith and Ziff, 1988). PD2 seems to be involved in the association of Ela with various cellular proteins, including the retinoblastoma gene product (an anti-oncogene product) (Yee and Branton, 1985; Whyte *et al.*, 1988b; Egan *et al.*, 1988) and this region can be functionally substituted by a structurally related 18 amino acid portion of SV40 T antigen (Moran, 1988). Sequences outwith the conserved domains have also been reported to be

involved in transformation (Whyte et al., 1988a; Velcich and Ziff, 1988), the rapid turnover of Ela (Slavicek et al., 1988) and nuclear localization (see Figure 9).

PD3 is a 46 amino acid region unique to the larger Ela protein and is essential for transactivation. A synthetic 49 amino acid oligopeptide spanning PD3 acts as an efficient activator of Ela inducible promoters in vitro and when injected into cells in the absence of de novo protein synthesis (Lillie et al., 1987; Green et al., 1988). This region encodes a zinc binding finger and single amino acid substitutions in the conserved cysteine residues abolish the transactivation activity of both the full-length polypeptide and the synthetic 49 amino acid peptide (Culp et al., 1988; Green et al., 1988). Ela, however, is not a DNA binding protein (Ferguson et al., 1985) so this transactivating region would seem to function by another mechanism.

Although the Ela transforming regions correlate more closely with repression than with transactivation a recent report suggests that the transformation and repression activities can be separated, and that mutants incapable of repression can still cooperate with Ras protein to induce transformation (Velcich and Ziff, 1988). The mutants still retain intact PDs 1 and 2, so it would seem that no single domain is responsible for repression function.

(ii) The mechanism of Ela transcriptional regulation.

Ela gene products stimulate transcription from a number of viral and cellular genes, but although Ela mediated transactivation has been studied intensely the mechanism of Ela function is still unclear.

Ela does not bind directly to DNA (Ferguson et al., 1985) nor have any promoter sequences required specifically for Ela responsivity been detected. It seems therefore that Ela mediates its activity through the promoter sequences necessary for uninduced transcriptional activity. The factors binding to regulatory elements upstream of Ela responsive genes have been studied in depth, but although Ela seems to modulate the binding activity of some of these factors (in the absence of de novo protein synthesis) no common mechanism for Ela activation has been revealed

(reviewed Jones et al., 1988). The ability of a small 49 amino acid subdomain of Ela to mediate transactivation (Lillie et al., 1987; Green et al., 1988) contrasts markedly with the activity of the short acidic activating regions of yeast transcription factors, which only function when fused to a DNA binding domain (Section 1E3.3). The Ela activating region is neither acidic nor DNA binding and may function through protein/protein interactions with cellular transcription factors, mediated by the potential zinc finger binding domain. It is intriguing that the activating domain of Ela is a very potent specific transactivator when fused to the GAL4 protein DNA binding domain, but still retains some activating activity on promoters lacking the GAL4 binding site UAS_{GAL} (Lillie and Green, cited in Ptashne, 1988).

It has been suggested that Ela may interact with and perhaps modify individual transcription factors to modulate their DNA binding activity. This modification could involve protein phosphorylation, and Ela mediated stimulation of transcription by RNA polymerase type III has been shown to involve the conversion of TFIIIC to a more potent DNA binding form associated with phosphorylation of this protein (Hoeffler et al., 1988). Ela activation has also been observed to function through an upstream promoter element involved in response to induction by the cAMP-dependent protein kinase II pathway. However, Ela activation was not protein kinase II dependent and it was suggested that Ela acivation might be achieved by directly or indirectly phosphorylating the transcription factor binding to this UPE by an alternative mechanism (Sassone-Corsi, 1988).

Factors responsible for the higher transcriptional activity of extracts of adenovirus infected cells co-fractionate with TFIID (Leong et al., 1988) and it has been suggested that Ela may exert its effect on RNA polymerase II transcription directly or indirectly through TFIID. Indeed the TATA box has been implicated in the mechanism of Ela mediated transactivation by two independent studies which have shown that the TATA box is the only promoter element involved in the response to Ela (Wu et al., 1987; Simon et al., 1988). Ela would appear to mediate

transactivation preferentially through a subset of TATA box sequences, with the hsp70 TATA box responding to E1a transactivation whilst the SV40 early promoter TATA box does not. This suggests that E1a may only interact with a subset of a family of TFIID factors (Simon et al., 1988). (A similar situation may be found for the HSV transactivator Vmw175, Everett, 1988b). In these experiments transactivation seemed to be mediated directly through the TATA box sequence in the absence of upstream regulatory regions. It is likely, however, that E1a does interact with or modify factors binding to upstream regulatory elements, such as E2F (Kovesdi et al., 1986; Yee et al., 1987).

The E1a protein is also able to repress transcription from a variety of cellular and viral enhancers (including the E1a enhancer), a function that is distinct from its transactivation activity. (Borrelli et al., 1984; Hen et al., 1985; Hearing and Shenk, 1985; Velcich and Ziff, 1985). The mechanism for this repression is unknown, but it seems to be fairly nonspecific.

3.5 A general mechanism for transactivation: the importance of the TATA box.

The TATA box is found upstream of the majority of class II promoters and is bound by the essential transcription factor TFIID (Sections 1E1 and 1E2.1). It appears to be mechanistic component of the promoter and not a regulatory element per se. However, as reviewed in the previous sections TFIID and its TATA box binding activity seems to be a common target for the action of eukaryotic transcriptional activators, including:

- the acidic activating regions of GAL4, GCN4, etc.
  (Horikoshi et al., 1988a; Lin et al., 1988)
- the PrV immediate early protein
  (Abmayr et al., 1988)
- the cellular transcription factor ATF
  (Horikoshi et al., 1988b; Hai et al., 1988).

In addition the TATA box appears to be the only identifiable important promoter element for the transactivation of a number of promiscuous viral transactivators (E1a: Section 1E3.4; Vmw175: Section 1D2.1; PrV IE180: Section 1D2.1).

Interactions between transcription factors bound at upstream promoter elements and TFIID seem to change the conformation of TFIID bound at the TATA box region, extending the region of DNA covered by the protein. It appears that these interactions may have to be mediated through a transcription factor bound close to the TATA box, but that more distally bound transcription factors can interact synergistically with this proximal factor, (Lin et al., 1988).

If TFIID (or indeed RNA polymerase or associated factors) is a direct target for transactivating interactions by a multitude of activating proteins, this would imply that the interactions between transcription factors and the responsive TFIID are fairly non-specific. The acidic or glutamine rich activating regions of many transactivators could provide such a non-specific signal.

How the activity of transcription factors themselves is controlled is an equally important question. This could be controlled at the level of synthesis (transcription) or by modulating the activity of constitutive transcription factors. Phosphorylation would appear to be an important mechanism for modulating the activity of at least some transcription factors. Phosphorylation of the serum response (transcription) factor is required for DNA binding (Prywes et al., 1988), and phosphorylation also modulates the DNA binding activity of the RNA polymerase III transcription factor TFIIIC (Hoeffler at al., 1988). Alternatively phosphorylation may modulate the activation activity of some transcription factors since both phosphorylated and non-phosphorylated forms of the heat shock transcription factor can bind to DNA (Sorger and Pelham, 1988). Other forms of post translational modifications including GlcNAc glycosylation may be important for regulating the activity of transcription factors (Jackson and Tjian, 1988). The direct liganding of cellular messengers such as the steroid hormones provides an important means of controlling the activity of the hormone receptors superfamily (Section 1E3.2).

4. Mechanisms for the repression of transcription.

Whilst the activation of transcription is probably the major level of gene regulation in eukaryotic cells, repression of RNA polymerase II mediated transcription also has a role in regulating gene expression and activation. Although several examples of transcriptional repression have been documented little is known about how repression is mediated and it is likely that repression can function at several different levels.

"Repression" can be mediated by blocking or preventing transcriptional activation. The activity of the unliganded steroid hormone receptors appears to be inhibited by being bound by cytoplasmic heat shock factors which prevent nuclear localization and DNA binding (Picard and Yamamoto, 1987; Denis et al., 1988; Section 1E3.2). A similar control mechanism is exerted on the transcription factor NF_kB which is bound by an inhibitory factor, I_kB, in the cytoplasm, and only released in an active form upon phorbol ester activation (Baeuerle and Baltimore, 1988). The yeast repressor protein GAL80 also functions to inhibit transcriptional activation by interacting with an activating protein, GAL4 (see Section 1E3.3). Association of GAL80 with GAL4 does not prevent GAL4 binding to its cognate upstream activating sequences, UAS_{GAL}, but the activating surfaces on the GAL4 protein do appear to be masked (Ma and Ptashne, 1987b). If an acidic activating region is fused onto GAL80, this protein, in complex with GAL4, now functions as an activator of transcription (Ma and Ptashne, 1988). This novel activator protein functions in an analogous manner to HSV Vmw 65 by associating indirectly with target promoters through the formation of a complex with a site specific DNA binding protein (Section 1C2.2).

The steroid hormone receptor superfamily forms an interesting group of transcription factors in that they can repress as well as activate specific promoters at the transcriptional level. As discussed above (Section 1E3.2) repression may be mediated by a variety of mechanisms. In some cases repression appears to operate through binding to elements related to their activating sequences (Glass et al., 1988) and in other cases appears to be mediated through an interaction with other transcription factors and to

function independently from the receptor DNA binding activity (Adler et al., 1988).

It has also been proposed that repression can be mediated by cellular factors which bind to upstream regulatory elements in competition with activating transcription factors. Separate DNA binding nuclear factors have been identified which seem to bind to a GRE and a CCAAT-box element in the histone H2B promoter. These proteins may mediate repression by preventing the activating transcription factors from binding to their cognate upstream sequences (Langer and Ostrowski, 1988; Barberis et al., 1987). It has also been suggested that a protein specific for binding to TA rich sequences may regulate the recognition of certain TATA boxes by competing for TFIID binding (Hobson et al., 1988).

The adenovirus Ela protein is also capable of repressing enhancer mediated transcriptional activation (Section 1E3.4). The mechanism for is obscure and appears to be fairly non-specific. Repression is not dependent on DNA binding, nor mediated by the same region of the protein involved in the activation of transcription, and presumably operates through protein/protein interactions or modifications of other transcription factors. It has been proposed that Ela may mimic a cellular factor with similar negative function in undifferentiated embryonal carcinoma cells (Imperiale et al., 1984). The SV40 early gene product T antigen is able to repress SV40 transcription by specifically binding to its own promoter region, but it has recently also been shown that T antigen can repress activation of transcription by the celluar transcription factor Ap2. This repression does not require DNA binding by T antigen, but appears to be the result of a direct interaction between T antigen and Ap2 (Mitchell et al., 1987). The function of viral repressors could have some relationship to the phenomenon of "squelching" proposed by Gill and Ptashne (1988) where overexpression of activating domains seems to cause general transcriptional repression by competing for essential components of the transcriptional apparatus.

As discussed earlier in this introduction (Section

lDl.5) HSV-1 Vmwl75 is a specific repressor of transcription from its own promoter, and this activity is dependent upon the Vmwl75-DNA binding site at the IE gene 3 cap site. Vmwl75 may act like a classical bacterial repressor and the strong DNA binding site present at the cap site function as an "operator", with tightly bound Vmwl75 interfering with promotion through this region.

SECTION 1F: AIMS OF THE RESEARCH PRESENTED THIS THESIS.

The initial aim of the work described in this thesis was to define the regions of the HSV-1 IE protein Vmw175 involved in functional activity by performing a detailed mutational analysis of a plasmid-borne copy of the gene, and assaying the activity of the mutants in transient transfection assays. Four phenotypes of Vmw175 were investigated, transactivation of an early promoter, repression of the IE3 promoter, cellular localization and site specific DNA binding. It was hoped to be able to correlate these results with known structural features of this protein based on its predicted amino acid sequence and homology with other herpes transactivators. In particular it was hoped to determine whether transactivation and repression were separable functions of the protein and whether DNA binding activity correlated more strongly with one transcriptional activity than the other.

Having completed this analysis of Vmw175 function in transiently based expression experiments it was planned to recombine some of the more interesting plasmid-borne mutants back into the virus genome in order to investigate their behaviour in the virally infected cell.

CHAPTER 2: MATERIALS AND METHODS

SECTION 2A: MATERIALS

1. Cells

(i) BHK-21 clone 13 (MacPherson and Stoker, 1962), a fibroblastic cell line derived from baby hamster kidney cells, was generally used for growth of virus stocks and viral gene expression experiments.

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

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

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

(v) M64A cells (obtained from Dr.N.D.Stow of this Institute) express HSV-1 Vmw175 and were used to complement the growth of viruses carrying lethal mutations in IE gene 3. M64A cells are equivalent to the cell line M65 described by Davidson and Stow (1985) and are a mixed cell line derived from BHKtk$^-$ cells cotransformed with the HSV-1 large *Hin*dIII-*Xho*I subfragment* of *Xho*IC, and the HSV-1 thymidine kinase (*tk*) gene and selected for thymidine kinase expression. (*This subfragment is deleted for *ori*$_S$).

2. Tissue Culture Media

(i) BHK cells were grown in Glasgow Modified Eagle's Medium (GMEM) (Busby *et al.*, 1964) supplemented with 100 units/ml penicillin, 100 ug/ml streptomycin, 0.2ug/ml amphotericin, 0.002% phenol red and 10% calf serum or newborn calf serum. (= EC10)

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

(iii) Flow HeLa cells were grown in supplemented GMEM as

for BHKs but with 10% foetal calf serum replacing the calf serum. (= EF10)

(iv) Vero cells were also grown in EF10.

(v) M64A cells were grown in EF10 supplemented with 1ng/ml Aminopterin, 5ng/ml Thymidine, 15ng/ml Hypoxanthine and 15ng/ml glycine. (= HAT medium)

(vi) Viral titrations were overlaid with GMEM supplemented as above and with 5% human serum (EH5), or with 0.6% Noble agar in GMEM without antibiotics or phenol red and with the appropriate serum.

3. Viruses

All viruses used in this study were derivatives of the HSV-1 Glasgow strain 17 syn$^+$, which forms non-syncitial plaques on BHK cells (Brown et al., 1973). Derivative in1411 which produces no Vmw175 (Russell et al., 1987b) was constructed by inserting an XbaI linker encoding stop codons in all three reading frames into the PvuII site of both copies of IE gene 3. The 17$^+$ derivative dl1403 (Stow and Stow, 1986) has a large deletion created within both copies of IE gene 1 and produces no Vmw110.

4. Bacteria

All plasmids used in this study were propagated in E.coli K12 strain HB101 (F$^-$, ramC1, proA2, galK2, strA, recA; Boyer and Roulland-Dussoix, 1969).

5. Bacterial Culture Media

All strains of bacteria were grown on L-broth (177mM NaCl, 10g/l Difco Bactopeptone, 5g/l yeast extract (pH7.5 prior to sterilization). Agar plates contained 1.5% (w/v) agar in L-broth and where appropriate the medium was supplemented with 100ug/ml ampicillin.

6. Plasmids

The following plasmids were kindly provided by the acknowledged authors.

p175 (Everett, 1987b) The HSV-1 IE3 gene is cloned on a BamHI-SstI fragment (HSV-1 coordinates 125.1-133.0) under the control of the SV40 early promoter and enhancer (SV40

coordinates 5171-346).

pl11 (Everett, 1987b) The HSV-1 IE1 gene is cloned on a HpaI-SstI fragment (HSV-1 coordinates 120.4-125.1).

p63 (Everett, per.comm.) The HSV-1 IE2 gene is cloned on a BamHI-SstI subfragment of the plasmid pGX152 (EcoRIc) (Everett, 1986).

pIE3CAT (Stow et al., 1986) The promoter of the HSV-1 IE3 gene is cloned on a HindIII-BamHI fragment (-331-+26) fused to the CAT gene in a vector derived from pBLW2 (Gaffney et al., 1985).

pgDCAT (Everett, 1986) The promoter of the gene encoding gD (US6) is cloned on a SstI-HindIII (-392-+11) fragment fused to the CAT gene in the vector pBLW2 (Gaffney et al., 1985).

pGEM-2 (Promega Biotech, Madison, Wis., USA) A dual T7/SP6 RNA polymerase expression vector.

pGEMTIF (Ace et al., 1988) The HSV-1 gene encoding Vmw65 cloned under the control of the T7 promoter in pGEM-2 (see Figure 13a).

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

pRED112 (Everett, 1983) Plasmid encoding a 5'-truncated version of the gD promoter with a XhoI linker inserted at -109.

pGX156 Contains the HSV-1 EcoRI h fragment spanning U_S.

The structures of the plasmids pl11, pl75 and pgDCAT are shown in Figure 10.

7. Reagents

Oligonucleotides were purchased from PL Biochemicals or synthesized in this Institute by Dr. John McLauchlan using a Biosearch 8600 DNA Synthesizer.

Most analytical grade chemicals were purchased from BDH Chemicals UK or Sigma Chemical Co.; TEMED and ammonium persulphate were obtained from BIO-RAD Laboratories; caesium chloride, acrylamide, sodium hydroxide and boric acid were obtained from Koch-Light Laboratories; nitrocellulose paper from Schleicher and Schuell Inc.; 0.25mm silica gel TLC plates were obtained from Camlab; unlabelled dNTPs were from PL Biochemicals; Tris was supplied by Boehringer Mannheim; double stranded poly dI:poly dC and poly $d(N)_6$ were

PLASMIDS EXPRESSING VMW110 AND VMW175

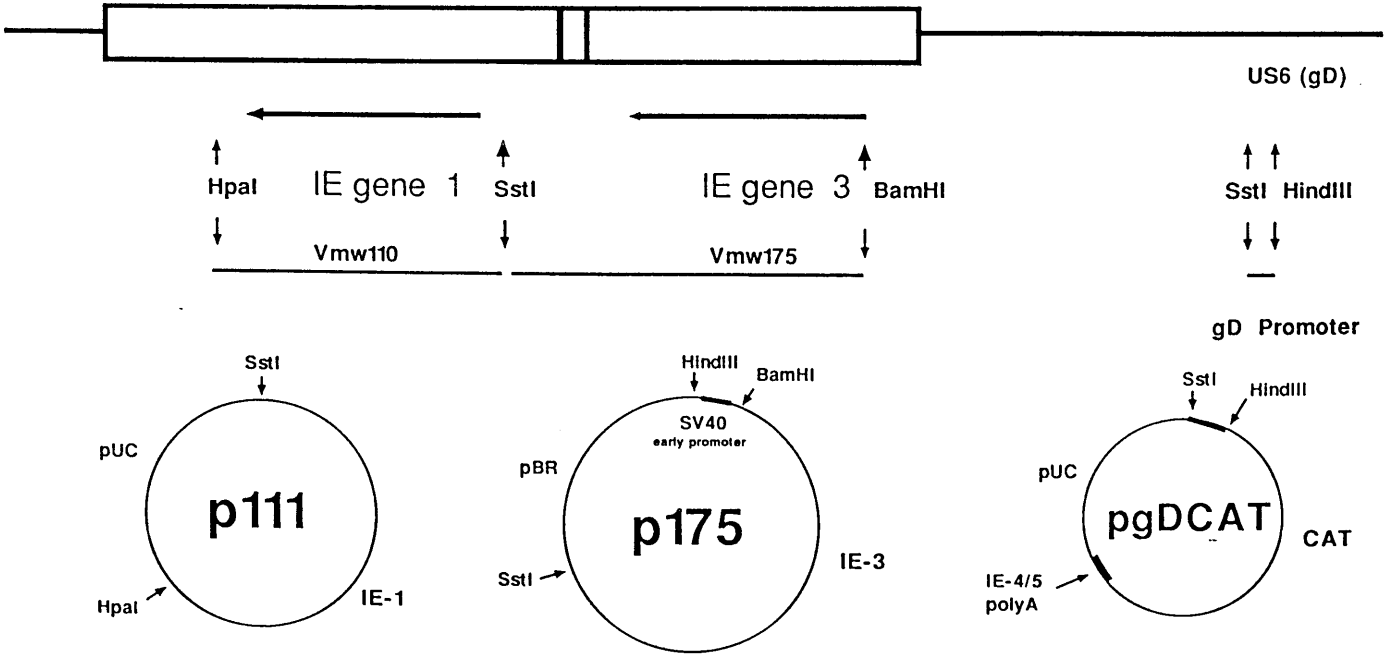


Figure 10: Structure of plasmids expressing Vmw110 and Vmw175. (Reproduced from an original diagram by RD Everett). The positions of the gD (US6), IE1 and IE3 genes are shown on a portion of the HSV-1 genome (see Figures 2 and 3). The structures of the plasmids p111, p175 and pgDCAT are detailed. p111 carries IE gene 1 cloned on an HpaI-SstI restriction fragment from IR_L. IE gene 3 is cloned on a BamHI-SstI fragment spanning IR_S and IR_L in the plasmid p175. The BamHI site is 26bp downstream of the normal transcriptional start site; in p175 transcription is driven by the SV40 early promoter and enhancer. The gD promoter is cloned on a SstI-HindIII (-392-+11) fragment and fused to the CAT gene in a vector derived from pBLW2 (Gaffney et al., 1985).

purchased from Pharmacia; Formamide from Fluka; Actinomycin D was from Merck Sharp and Dohme and Cycloheximide was from Sigma. Reagents for in vitro transcription were from Promega Biotech and Pharmacia, Rabbit reticulocyte lysate was from Amersham.

8. Radiochemicals

Biochemical radionuclides were obtained from NEN DuPont at the following specific activities:

5' [alpha-^{32}P] dNTPs, 3000 Ci/mmol (10uCi/ul)

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

^{14}C Chloramphenicol, 45mCi/mmol

L-^{35}S-Methionine, around 800mCi/mmol (from Amersham plc)

^{32}P Orthophosphate, 200mCi/mmol

9. Enzymes

DNase, RNase and lysozyme were supplied by Sigma; Proteinase K, Klenow fragment DNA Polymerase (LFP), DNA polymerase holoenzyme, T4 polynucleotide kinase and T4 DNA ligase were from Boehringer Mannheim; Bacterial Alkaline Phosphatase (BAP) was obtained from Worthington and purifed by M.Dunlop; most restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs.

10. Antibodies

The anti-Vmw175 monoclonal antibody 58S was a kind gift from Dr. M.Zweig (Showalter et al., 1981). Horse-radish peroxidase conjugated anti-mouse Immunoglobin and Fluoroscein conjugated swine anti-mouse imunoglobulin were purchased from BIO-RAD. Antipeptide antisera, listed below, were produced in this institute, and kindly supplied by Dr. M.Frame.

14711 Anti-carboxyl terminus Vmw110

11666 Anti-amino terminus Vmw175 (Palfreyman et al., 1984)

18817/18818/19825/19826 Anti-carboxyl terminus Vmw175

18815/18816 Anti-carboxyl terminus Vmw63

13963 Anti-carboxyl terminus HSV-1 US9 ORF

14996 Anti-carboxyl terminus RR2 (38kDa ribonucleotide reductase subunit)

2180V Rabbit antiserum raised against undefined fusions of Vmw175 (possibly including amino terminal regions) and E.coli beta-galactosidase (kindly supplied by Dr. A.Buchan of Birmingham University).

11. Solutions

ABTS Buffer: 100mM Na_2HPO_4, 80mM Citric acid, pH 4.0

5X BALI buffer: 50mM Tris.HCl (pH7.6), 25mM $MgCl_2$, 0.5mM$(NH_4)_2SO_4$, 5mM 2-mercaptoethanol, 500ug/ul BSA, 25mM KCl,

2X BBS: 50mM BES, 280mM NaCl, 1.5mM Na_2HPO_4, (pH6.95)

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

CLB (Cell lysis buffer): 0.5% SDS, 20mM TrisHCl (pH7.5), 2mM EDTA

50X Denhardt's: 1% Polyvinylpyrrolidone, 1% BSA, 1% Ficol

20X dNTPs: 1mM dATP, dCTP, dGTP, and dTTP.

ELISA coating mix: 1% ovalbumin, 1% BSA, 0.1% Tween20 in PBS. (With or without 0.05% Sodium Azide).

5X Gel Loading buffer: 1X TBE, 1% SDS, 50% glycerol, plus bromophenol blue.

Gel soak I: 200mM NaOH, 600mM NaCl,

Gel soak II: 1M Tris.HCl (pH7.5), 600mM NaCl

Gel elution buffer: 0.5M Ammonium Acetate, 20mM Tris.HCl (pH7.5), 2mM EDTA

Giemsa stain: 1.5% suspension of Giemsa in glycerol, heated at 56^{o}C for 2h and diluted with an equal volume of methanol.

200X HAT: 0.2mg/ml Aminopterin, 1mg/ml Thymidine, 3mg/ml Hypoxanthine, 3mg/ml Glycine, pH to ~10 to dissolve.

2X HBS: 280mM NaCl, 50mM Hepes, 1.5mM Na_2HPO_4, pH to 7.12.

Hybridization mix: 6X SSC, 5X Denhardt's, 0.1% SDS, 50ug/ml denatured calf thymus DNA

HZ Stop: 0.3M NaAcetate (pH5.5), 0.1mM EDTA, 25ug/ml tRNA

5X Kinase buffer: 350mM Tris.HCl (pH7.5), 50mM $MgCl_2$, 25mM DTT

5X Ligase buffer: 250mM Tris.HCl (pH7.5), 50mM $MgCl_2$, 100mM DTT, 50ug/ml BSA

5X Nae buffer: 100mMNaCl, 50mM Tris.HCl pH8, 50mM $MgCl_2$, 25mM 2-mercaptoethanol, 500ug/ml BSA

NE buffer A: 10mM HEPES (pH7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5% NonidetP40

NE buffer C: 20mM HEPES (pH7.9), 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.5mM PMSF, 0.5mM DTT

10X NT buffer: 0.5M Tris.HCl (pH7.5), 100mM $MgCl_2$, 100mM DTT, 500ug/ml BSA

NT Stop: TE plus 1% SDS

PBS-A: 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 (pH7.2)

PBSc: PBS-A plus $CaCl_2H_2O$ and $MgCl_26H_2O$ at 1g/l.

5X Primer extension mix: see Section 2B22.4

5X Pst buffer: 30mM Tris.HCl(pH7.8), 30mM $MgCl_2$, 250mM NaCl, 30mM 2-Mercaptoethanol

BRL REact™ Buffers (1X concentration):

REact 1: 50mM Tris.HCl (pH8), 10mM $MgCl_2$

REact 2: 50mM Tris.HCl (pH8), 10mM $MgCl_2$, 50mM NaCl

REact 3: 50mM Tris.HCl (pH8), 10mM $MgCl_2$, 100mM NaCl

REact 4: 20mM Tris.HCl (pH8), 5mM $MgCl_2$, 50mM KCl

REact 10: 100mM Tris.HCl(pH8), 10mM $MgCl_2$, 150mM NaCl

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

SDS boiling mix: 1ml SGB, 0.8ml 25% SDS, 0.5ml 2-mercaptoethanol, bromophenol blue.

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

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

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

5X SmaI buffer: 30mM Tris.HCl (pH8), 30mM $MgCl_2$, 100mM KCl, 30mM 2-mercaptoethanol, 500ug/ml BSA

STET: 8% Sucrose, 5% Triton X-100, 50mM EDTA (pH8),60mM Tris.HCl (pH8).

Sucrose gradient solution: 200mM NaCl, 25mM Tris.HCl (pH7.5), 5mM EDTA, 0.15 Sarkosyl, 5ug/ml Ethidium Bromide, plus 5% or 20% Sucrose.

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

TBS: 25mm Tris.HCl (pH7.5), 137mM NaCl, 5mM KCl, 0.7mM $CaCl_2$, 0.5mM $MgCl_2$, 0.6mM Na_2HPO_4, pH to 7.4 with HCl.

TE: 10mM Tris.HCl, 1mM EDTA, pH 8.0

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

TEP: 1mM Tris.HCl (pH7.5), 0.05mM EDTA.

T4 Pol Buffer: 33mM Tris Acetate (pH7.9), 66mM Na Acetate, 10mM MgAcetate, 100mg/ml BSA, 0.5mM DTT.

Trypsin: 0.25% w/v Trypsin dissolved in Tris-Saline

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

Zweig's extraction buffer: 100mM TrisHCl (pH8), 10% glycerol, 0.5% NonidetP40, 0.2mM PMSF

SECTION 2B: METHODS

1. Tissue Culture

BHK cells were routinely passaged in 850cm^2 roller bottles seeded with approximately 5x10^7cells at 37^oC in 100ml EC10 and an atmosphere of 5% CO_2 in air. Confluent monolayers were harvested in 20ml EC10 after two Versene washes and brief trypsinization with Trypsin:versene (1:4). Cells were resuspended by pipetting and remained viable for at least five days stored at 4^oC.

Vero, HeLa and M64A cells were routinely passaged in 175cm^2 (Nunclon) flasks in appropriate medium and harvested

in 10ml. Cells did not remain viable at 4°C and for long term storage harvested cells were pelleted and resuspended in medium plus 5% glycerol and 15% foetal calf serum, aliquoted and frozen slowly to -140°C. Recovery was by rapid thawing and resuspension in growth medium.

2. Preparation of Stocks of Infectious Virus

An 80% confluent monolayer of BHK or M64A cells was seeded with 10^6 pfu of virus per roller bottle in 40ml EC5 or HAT medium (a moi of 0.003pfu/cell) and incubated for 2-4 days until the cells exhibited obvious CPE. The cells were shaken into the medium and pelleted at 3K rpm/ 15 min/ 4°C. Cell associated virus (CAV) was prepared by sonicating the pellet in 2ml supernatant. Cell released virus (CRV) was pelleted from the supernatant at 12K rpm/ 2 hours/ 4°C and gently resuspended in 2ml supernatant and sonicated. Sterility checks were performed by streaking virus preparations on blood agar plates and incubating at 31°C for 5 days. Virus stocks were stored at -70°C.

3. Titration of Virus Stocks

Appropriate indicator cells were seeded at 10^6 cells per 35mm plate in 2.5ml medium. The following day cells were infected with serial 10-fold virus dilutions in 0.3ml medium; after 1h absorption time (gently mixing every 10 minutes) plates were overlaid with 2ml EH5, to prevent secondary plaque formation, and incubated for 2-3 days prior to fixing with Cidex and staining with Giemsa stain. The plates were washed after 15 minutes and plaques counted using a dissection microscope. Usually titrations on M64A cells were overlaid with 0.6% Noble agar in HAT medium lacking phenol red, and plaques visualized by staining living cells for 6h with neutral red.

4. Preparation of Cell Released Viral DNA

CRV were pelleted as described in 2 above and resuspended in 1ml TE plus 100mM NaCl. SDS was then added to 1% v/v and viruses disrupted by gentle inversion at 37°C. DNA was then extracted by serial 30 minute extractions, with gentle inversion (2x phenol, 1x phenol/chloroform, 1x

chloroform). DNA was precipitated with .05 vol 5M NaCl and 2.5 vol ethanol, washed twice, dessicated and resupended overnight in TE. DNA was quantitated on agarose gels compared to known standards and stored at -20°C.

CRV DNA was prepared on a miniature scale from 35mm plate harvests in a similar manner, but disrupting the virus particles in 20 mM Tris pH7.5, 100mM NaCl, 2mM EDTA, 0.2% SDS, 0.25mg/ml proteinase K.

5. Preparation of Total Infected Cell DNA

10mm linbro wells were seeded with about $4x10^5$ permissive cells and infected the following day with half of a picked plaque or a moi of 0.001 pfu/cell. After 2-3 days when cpe became apparent, the supernatant was removed and stored as CRV stock. 0.2ml of Cell Lysis Buffer (CLB) containing 0.25mg/ml proteinase K was added to the cell layer and incubated at 37°C for 3-5 hours. The glutinous extract was transferred to 1.5ml Eppendorf tubes and 15ul 5M NaCl added. Serial 30 minute phenol then chloroform extractions were performed, with gentle inversion; followed by precipitation with 2.5 vols ethanol, resuspension in 0.3M Na Acetate pH5, a further ethanol precipitation, one wash with 70% ethanol, dessication and finally resuspension overnight at 4°C in 50ul TE.

6. Plaque Purification of Virus

Permissive cells were infected with virus dilutions as for virus titrations, but overlaid with 2ml molten 0.6% Noble agar in growth medium (100ml 1.3x Eagles A, 20ml Eagles B without phenol red, 30ml 3.2% Noble Agar, 5ml appropriate serum, with or without HAT). After the agar set, plates were incubated for 2-3 days at 37°C. Plaques were visualized by dark field microscopy or by staining living cells with neutral red for 6 hours. Isolated plaques were picked using a sterile Pasteur pipette to draw up a plug of agar and infected cells and then ejecting this into 200ul of medium. 100ul was used to infect a linbro well to make CRV and infected cell DNA.

7. [^{32}P]orthophosphate Labelling of Viral DNA

The technique of Lonsdale (1979) modified by Brown et al., (1984) was used. Cells were seeded at $5x10^5$ cells per linbro well in phosphate-free Eagles medium plus 1% calf serum (PIC). The following day wells were infected with 10 pfu/cell virus, and after absorption the cells washed twice with PIC and overlaid with 450ul PIC and incubated at 31°C for 2h prior to addition of 50ul of PIC containing 2MBq of [^{32}P]orthophosphate. After incubation at 31°C for 2 days cells were harvested by adding 0.5ml 5% SDS to each well for 5 mins at 37°C. Disrupted cells were then scraped into 10ml tubes and phenol extracted gently for 10 minutes before ethanol precipitating the DNA, washing, drying and resuspending in 200ul H_2O; 20-30ul aliquots were digested to analyse on agarose gels. After running gels as described below (Section 19.1) Gels were dried down at 80°C and autoradiographed.

8. Analysis of Virus Induced Polypeptides

8.1 Immediate-early polypeptides

IE extracts were generally made at 38.5°C. $2x10^5$ BHK cells on 50mm Petri dishes were pretreated with 100ug/ml cycloheximide in growth medium (EC10/CHX) for 15-30min prior to infection at a moi of 5-20 pfu/cell, also in EC10/CHX. After a 1h absorption the cells were washed and overlaid again with EC10/CHX. At 5h p.a. cells were washed 4x with prewarmed PBSc, with the final wash containing 2.5ug/ml Actinomycin D. 50uCi of [^{35}S]methionine was then added for a one hour incorporation period before a final wash with PBSc prior to harvesting at 4°C for 1h in 400ul Zweig's extraction buffer. The lysate was sonicated and debris spun out with a 5min microfuge spin. Extracts were stored at -70°C.

8.2 Long labelling of polypeptides

Sub-confluent BHK cells (10^6 cells on a 50mm Petri dish) in methionine-free EC10 were infected at a moi of 10 and after a 1h absorption the cells washed and then overlaid with methionine-free EC10 plus 50uCi [^{35}S]methionine. The

cells were harvested after an overnight incubation and protein extracts made as described in section 8.2 above.

8.3 Pulse labelling of polypeptides

Sub-confluent BHK cells (10^6 cells on a 50mm Petri dish) were infected at a moi of 10 and overlaid with EC10 after a 1h absorption. At the appropriate time p.a. the cells are washed with PBSc and labelled for 30min with PBS containing 50-100uCi [^{35}S]methionine. Cells are then washed and harvested as above.

8.4 SDS polyacrylamide gel electrophoresis

[^{35}S]methionine labelled poypeptides were resolved on 7.5% or 10% single concentration gels crosslinked with 1 part in 12 (w/w) N-N'-methylenebisacrylamide in resolving gel buffer. Gel plates were carefully washed with Decon, 1mM HCl and ethanol. The resolving gels were made up as follows:

	7.5% gel		10% gel	
	narrow	wide	narrow	wide
30% (a+b) stock	9ml	13.5ml	12ml	18ml
RGB	9ml	13.5ml	9ml	13.5ml
distilled water	18ml	27ml	15ml	22.5ml
10% APS	200ul	200ul	300ul	300ul
TEMED	20ul	20ul	30ul	30ul

The resolving gel was overlaid with butan-2-ol and allowed to set. After washing, the stacking gel (5% a+b, 0.25xSGB) was prepared and overlaid, wells were created with a teflon comb.

Protein samples were boiled for 2min in 1 or 2 volumes of SDS boiling mix prior to loading. Gels were run at 0.45mA/cm^2 for 4h or 0.09mA/cm^2 for 18h in freshly prepared SDS tank buffer. Gels were fixed for 1h in methanol:acetic acid:water (50:7:50) before drying down under vacuum at 80^{o}C onto a sheet of Whatman 3mm paper and exposing to Kodak X-Omat S XS-1 film for 3-24h.

9. Transfection of Cells with DNA

9.1 Transfection of HeLa and Vero cells for CAT assays

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 4 ug of the CAT and pl11 plasmids, 8ug of the pl75 derivatives in the transactivation studies and variable amounts to titrate repression; pUC9 was used to equalize the amount of DNA within experiments. TEP buffer was added to the plasmid DNA to final volume of 140ul, then 20ul of 2M $CaCl_2$ added dropwise to the solution whilst vortexing. In turn this was added dropwise to 160ul of 2xHBS (or sometimes 2x BBS). After standing for 30min the precipitate was sprinkled onto the cell monolayers which were then incubated at 38.5°C. The cells were washed after 24 h and fresh medium replaced, after a further 24 h incubation at 37°C extracts were prepared by washing the cells in PBS-A then scraping into 3ml TEN. The cells were pelleted at 2K rpm/ 4°C/ 1min, then resuspended in 75ul 0.25M Tris pH 7.8 and sonicated. The sonicates were cleared by a 2min microfuge spin and stored at -20°C.

9.2 Transfection of HeLa cells for Protein Extracts

Transfections were carried out in exactly the same manner as described above (9.1) but the volumes increased 3 fold for 90mm plates or 7 fold for 135mm plates. 30 or 60ug of pl75 derived plasmids were used, with 15 or 30ug pUC9 as carrier.

9.3 Transfection of Cells with Viral DNA for Marker Rescue

BHK or M64A cells were tranfected on 35mm plates seeded at subconfluence. 0.5ug of intact viral DNA plus a 10 fold molar excess of linearized plasmid and 2-5ug calf thymus carrier DNA were precipitated as described above (9.1). After standing for 30min the medium was removed from the cells and the precipitate added, the cells were then incubated at 38.5°C for 4h, being overlaid with 2ml medium after 40min. The cells were then washed once with growth

medium and overlaid with 25% DMSO in HBS for 4min (for M64A cells 20% glycerol was used). The cells were then washed a final time and overlaid with growth medium. After 2-4 days incubation at 31 or 37°C and the monolayers exhibited extensive cpe, or plaques, the cells were scraped into the medium and sonicated to release infectious virus.

10. CAT Assays

Cell sonicates produced as described above (9.1) were assayed for chloramphenicol acetyl transferase (CAT) activity essentially as described by Gorman et al. (1982). Assay mixtures contained 1ul 50mM acetyl-CoA, 0.5ul stock [^{14}C]CAP (45uCi/mm) and 14ul H_2O together with 25ul neat or diluted sonicate. After incubation for 30 or 60min at 37°C the products were extracted with 200ul ethyl acetate, dried down and resuspended in 20ul ethyl acetate to spot onto thin layer chromatography (TLC) plates. TLCs were run in 95% chloroform/ 5% methanol, air dried and autoradiographed at room temp. The percentage conversion to product was determined by cutting out non-acetylated substrate and 3'-monoacetylated product from the TLC and counting scintillation in 2ml ecoscint. The protein concentration of the extract was determined by the method of Bradford (1976) (see section 11 below) in order to calculate the percentage conversion from substrate to product per ug protein. In the transactivation experiments the activity of the mutants was expressed as a percentage of the wild-type control; the results of the repression titrations were plotted and activity qualitatively compared to a wild-type control (as described in the Results Section 3C2).

11. Determination of Protein Concentration in Cell Extracts

The protein concentration of CAT extracts and nuclear protein extracts was determined by the method of Bradford (1976). 5-10ul of extract was made up to 100ul in 0.05M Tris.HCl pH7.8 and 1ml of Bradford's Reagent added and mixed. After a 15min incubation the OD at 595nm was measured in disposable plastic cuvettes by spectrophotometry. The protein concentration per ul extract was determined by constructing a standard curve using

standard concentrations of BSA (5-80ug) assayed as above, and OD plotted against concentration mg/ml.

12. Immunofluorescent Staining of Vmw175 in Transfected Cells

HeLa cells were seeded into 50mm Petri dishes and transfected with 10ug of pl75 and its derivatives as described above (9.1), washing and re-feeding after 24 h. After a further 24 h the cells were fixed and stained (essentially as described by Everett, 1987b). The cells were washed twice with 1%CS in PBSA then fixed for 10 minutes in 2% formaldehyde, 2% sucrose in PBSA. After 3 further washes with PBSA/1%CS the cells were permeabilized with 0.5% NP40, 10% sucrose, 1% CS in PBSA for 5min. After 3 further PBSA/1%CS washes the cells were stained with a 1/50 dilution in PBS of the anti-Vmw175 monoclonal antibody 58S (kindly supplied by M.Zweig; Showalter et al., 1981). After a further 3 PBSA/1%CS washes the cells were stained with fluorescein-conjugated swine anti-mouse immunoglobulin (1/30 dilution) before examining by u.v. fluorescent microscopy.

13. Preparation of Nuclear and Cytoplasmic Extracts for DNA Binding Studies

Nuclear extracts from cells infected or transfected as in section 9.2 above were prepared by a procedure modified from that of Dignam et al.,(1983). Cells scraped from 135 or 90mm culture dishes were washed in phosphate buffered saline (PBSc) and resuspended in 2 volumes NE buffer A without NP40. 0.5% NP40 was then added and the cells lysed on ice for 10 min. Nuclei were then pelleted by successive 2K/10min and 12K/20min spins. Proteins were eluted from the nuclei by incubating for 30 minutes on ice in 2 volumes buffer C before clearing with a 30 minute 15K spin. The supernatants were flash frozen and stored at -140^{o}C and retained their activity for at least 12 months.

In order to recover proteins from the cytoplasm some extracts were produced by lysing cells directly with buffer C plus 0.5% NP40. Nuclear binding activity was also recovered using this procedure and this much more rapid

protocol was used for many extracts where it was unimportant to distinguish between nuclear and cytoplasmic fractions.

14. Determination of the Relative Quantities of Vmw175 in Nuclear Extracts

5ul samples of each nuclear extract were assayed by the ELISA technique. Microtitre wells were coated overnight with extract plus 100ul ELISA coating mix with 0.05% sodium azide. A 1/5000 dilution in coating mix of the monoclonal antibody 58S was adsorbed to the coated wells for 1h shaking at 37°C and a 1/3000 dilution in coating mix of BIO-RAD horseradish peroxidase labelled goat anti-mouse IgG was bound to this at 20°C for 1h. Extensive washes with PBS/0.1% Tween 20 were carried out between all steps. The absorbance at 405 or 410nm was measured 10 minutes after addition of colourimetric substrate for peroxidase (ABTS) by spectrophotometry or on a plate counter.

15. Gel Retardation Assay

The binding of Vmw175 to the IE gene 3 cap site was assayed as described by Muller(1987). The 45bp Ava I/BamHI fragment spanning the IE3 cap site (-17 to +27) was end labeled by filling in using T4 DNA polymerase and purified on an 8% polyacrylamide gel and a Sephadex G-50 column. Binding reactions were generally carried out at 20°C for 20 minutes in a 20-30ul mix containing 1ug poly(dI).poly(dC), about 0.1ng probe (2000cpm) and 1-4ul nuclear extract (4-20ug protein) in 10mM TrisHCl pH7.6, 1mM EDTA, 0.1% NP40. Samples were resolved on 4% polyacrylamide gels (3.3% cross linker) run in 0.5x TBE, and dried down before autoradiography.

Antibody shifts were carried out by incubating the binding reaction for an additional 10-15 minutes after adding a 1/100 or 1/200 dilution of monoclonal 58S.

16. Large Scale Plasmid DNA Preparation

Isolated single colonies on plates selective for a plasmid-borne resistance marker were inoculated into 5ml L-broth and grown overnight. These were added to 350 or 700ml L-broth in 2l flasks and grown shaking at 37°C until

the cells reached an OD_{650} of approximately 0.8. Then chloramphenicol was added to a final concentration of 100ug/ml and the cultures left shaking overnight to amplify the plasmids.

Plasmid DNA was prepared by the "maxi-boiling" technique of Holmes and Quigley, 1981. Cells were pelleted at 10krpm/ 6min (Sorvall GS3 rotor) and resuspended in 17ml STET per 350ml culture. 2ml 10mg/ml lysozyme in STET was added for a 1 minute incubation prior to bringing the suspension to the boil for 45sec. The lysate was cleared with a 45min/ 20krpm/ 4°C spin (Sorvall SS34 rotor) and DNA precipitated from the supernatant by addition of 0.9vol isopropanol. After pelleting at 3krpm/ 4min/ RT (MSE coolspin) the drained pellets were resuspended in TE, and CsCl added to give a final density of 1.6g/ml and EtBr to 0.5mg/ml. The solution was cleared by spinning at 3krpm/ 10min/ 4°C after a 15min incubation on ice. The DNA was banded by spinning at 40krpm/ 15°C in the angled Ti50 or Type65 rotors (48+h) or the vertical TV865 rotor (16+h). The DNA was visualized by daylight or long-wave uv illumination and the lower, supercoiled plasmid DNA band recovered with a large bore needle and syringe, after removal of the upper, linearized chromosomal DNA band. The DNA was extracted twice with butan-1-ol (TE saturated), dialyzed against TE at RT for 3h, treated with 50ug/ml RNAase (1h/65°C), then 50ug/ml proteinase K plus 0.1% SDS (1h/37°C) before extraction with phenol/chloroform then chloroform and precipitation twice with ethanol. The DNA was lyophilized, resuspended in TE and its concentration determined by spectrophotometry (OD_{260} 1.0 = 50ug DNA/ml).

17. Miniprep Plasmid DNA Preparation

Single plasmid-transformed bacterial colonies were grown overnight in 5ml L-broth. 1.5ml stationary phase culture was pelleted 12krpm/ 15sec (benchtop minicentrifuge) and cells resuspended in 200ul STET. The cell suspension was immediately placed in a 100°C water bath for 45sec after addition of 5ul lysozyme (10mg/ml), then centrifuged at 12krpm for 10min. The pellet was removed and DNA precipitated and pelleted from the supernatant by addition

of 0.9vol isopropanol and spinning at 12krpm for 5min. The pellets were washed with 70% ethanol, dried and resuspended in 20ul TE. Miniprep DNA was stored at -20 or 4°C and 2-5ul was used for restriction enzyme analysis. (0.2ul 1mg/ml RNAase was added to digestions of miniprep DNA).

18. Restriction Enzyme Digests

Restriction enzyme (RE) digests were generally carried out in 20ul volumes of the appropriate RE buffer, using 0.5ug DNA and 1 unit of enzyme for 1h. Originally most digests were carried out in 1x Pst buffer, with the exception of REs requiring radically different conditions (see below). Laterly the BRL REact™ system has been used, with commercially supplied 10x REact buffers. The buffer conditions used for each enzyme are given below.

REact 1: AluI, NarI, RsaI
REact 2: AvaI, AvaII, BglI, BglII, BstEII, HindIII, PstI, SstI, SstII, TaqI, XbaI, XhoI, PvuII
REact 3: BamHI, BglII, EcoRI,
REact 4: HincII, SmaI
REact 10: SalI

NaeI and BalI were always digested in their own buffers; SmaI and BstNI were generally digested in Sma buffer; enzymes not listed under REact 2 or 3 above generally were digested under the makers specific conditions prior to introduction of the REact system. Digestions with TaqI and BstNI were carried out at 65 and 60°C, all others were at 37°C.

Preparative rather than diagnostic RE digestions used increased amounts of DNA and RE under similar conditions, in 20-50ul volumes.

19. Separation and Preparation of Purified DNA Restriction Fragments

19.1 Non-denaturing agarose gels

Slab gels of 0.4-1.5% agarose in 1x TBE (or 1x L buffer for Southern transfers) were run submerged at up to 12V/cm. Samples were loaded in 0.2 vol loading buffer. Gels were

run for 1-3h, until the dye almost ran off the end, and then stained for 10min in 1ug/ml EtBr and examined and photographed with short-wave uv transillumination (long-wave uv was used to reduce DNA damage when preparative gels were being analysed).

To purify a resolved DNA fragment from a gel the DNA was run onto a strip of DE-81 paper inserted in front of the desired band, (DE-81 paper was presoaked in 2.5M NaCl overnight, then stored in 1mM EDTA at 4^{o}C). After electrophoresing the fragment onto the paper it was eluted by pulverization and agitation in 1.5M NaCl/ 1x TE (2h/37^{o}C), strained through siliconized glass wool, clarified and then ethanol precipitated.

19.2 Non-denaturing polyacrylamide gels

In order to resolve accurately DNA fragments 40-400bp in length 4-8% polyacrylamide (acrylamide:bis-acrylamide, 19:1) gels were run in 1x TBE. Gels were prepared in 1.5mm thick vertical glass-plate sandwiches, and cross-linked with 0.001vol TEMED, catalysed with 0.01vol 10% APS. Samples were loaded as above and electrophoresed at a maximum voltage of 16V/cm, and DNA visualized by autoradiography or EtBr staining. ^{32}P-labelled DNA fragments were cut out of gels after detection by autoradiography and purified in one of two ways. Polyacrylamide strips containing fragments for sequencing were extruded through a punctured 0.4ml vial into a 1.5ml tube (benchtop minifuge/ 5min/ 12krpm). The DNA was eluted into TE by shaking overnight and filtered through glass wool before precipitation. Probes for gel retardation assays were eluted into gel elution buffer (2x 3h/ 45^{o}C incubations) after dicing the polyacrylamide strips. The supernatant was then purified on a Sephadex column (Section 2B19.5).

19.3 Denaturing polyacrylamide gels

0.35mm thick gels prepared to final concentration of 8-20% acrylamide (a:b, 19:1), 1x TBE and 7M urea were used to resolve the products of DNA sequencing reactions. The acrylamide solution was filtered through a 0.45um filter and degassed under vacuum prior to cross-linking. Lyophilized

samples were resuspended in 5ul formamide dyes mix, denatured at 90°C for 2min and run at 40W for 2-5h on gels which had been prerun for 30min at 40W. Gels were autoradiographed at -70°C with screens.

19.4 Sucrose gradients

Depending on the separation required 5-20% sucrose gradients were prepared in either 5 or 14ml polyallomer tubes using a peristaltic pump connected to a mixing chamber. 10-50ug of DNA was carefully overlaid onto the gradient in 100ul TE and the gradients centrifuged under appropriate conditions. (5ml tubes were spun in the AH650 rotor for 3-20h/ 30-40krpm/ 15°C, linear pl75 was separated from circular DNA in the TST41 rotor at 28krpm/ 16h/ 15°C.) DNA bands were visualized with long wave uv light and removed by side puncture. DNA was purified by phenol/chloroform extraction and ethanol precipitation.

19.5 Sephadex columns (gel exclusion chromatography)

Sephadex beads were pre-swollen by autoclaving in TE or H_2O. Columns were prepared in plastic 5ml pipettes with siliconized glass wool plugs and prerun with 20ml running buffer (TE plus 0.1% SDS was used for all columns except for gel retardation probes which were run with H_2O). Sephadex G-50 was used except for DNA fragments smaller than 20bp when Sephadex G-15 was used. Columns were loaded with 100-800ul sample (with or without bromophenol blue dye) and the fastest eluting fractions (detected by scintillation counting) taken as purified DNA.

20. <u>DNA Ligation</u>

DNA ligations were performed in 20ul 1x ligase buffer, with 0.5mM ATP, 50-500ng DNA and 1-2u T4 DNA ligase. DNA was frequently phenol and chloroform extracted, ethanol precipitated (with lug carrier tRNA) and resuspended prior to tranformation of competent bacteria.

21. <u>Preparation and Transformation of Competent E.coli</u>

A colony purified overnight culture of bacteria was diluted into fresh L-broth plus 10mM $MgCl_2$/ 10mM $MgSO_4$ and

aerated vigourously until reaching an OD_{540} of 0.3. Using sterile, ice cold, procedures throughout, cells were pelleted (3krpm/ 10min) resuspended in 1/4vol 0.1M $MgCl_2$ and immediately pelleted again (3krpm/ 10min). After resuspension in 1/20vol 0.1M $CaCl_2$ the cells were left for 45min on ice before again pelleting (3krpm/ 10min). The cells were resuspended in the same volume of 0.1M MOPS/ 50mM $CaCl_2$/ 20% glycerol and left 20min before aliquoting into 50-200ul and snap freezing and storing at -70°C.

22. Radioactive Labelling of DNA

22.1 5'-end labelling

To label DNA fragments at the 5'-end the 5'-phosphate was first removed by treatment with bacterial alkaline phosphatase (BAP) for 1h at 65°C in TE. The enzyme was rigourously removed with three phenol then chloroform extractions and the DNA precipitated to concentrate. The DNA was lyophilized and phosphoryl/ation was carried out in 10ul of 1x kinase buffer including 4 units T4 polynucleotide kinase and 10-100uCi 5'-[gamma^{32}P]dATP (45min/ 37°C). The labelled DNA was purified from unincorporated label by precipitation with lug carrier tRNA, running on Sephadex G-50 columns or by gel electrophoresis.

(Oligonucleotides were phosphorylated in a similar fashion but using 0.5mM cold ATP in the kination reaction. The reaction was stopped at 70°C.)

22.2 3'-end labelling

3'-ends of DNA fragments were labelled by filling in 5'-overhangs with either LFP or T4 DNA polymerase. Digests of 1-2ug of DNA were filled in after stopping the restriction enzyme at 65°C. LFP reactions were carried out in 1x Pst, REact 1 or REact 2 buffers, and used 1u enzyme incubated at RT for 20min with appropriate hot (5'-[alpha^{32}P])and cold dNTPs followed by a 20min chase with all four cold dNTPs. T4 DNA polymerase reactions differed in that it was incubated in its own buffer at 37°C for 1h. Both enzymes were inactivated at 65°C prior to second enzyme digestions or DNA purification.

22.3 Internal labelling by nick translation

100ng of plasmid DNA was radiolabelled (Rigby et al., 1977) by the addition of 5uCi each of all four 5' [alpha-^{32}P]dNTPs, 1 unit E.coli DNA polymerase holoenzyme and $5x10^{-7}$ug/ml DNAaseI in a final volume of 20ul 1x REact 1 buffer or 1x NT buffer. After 1h incubation at RT the reaction was stopped by addition of 100ul NT stop and labelled DNA purified on a Sephadex G-50 column (19.5).

22.4 Internal labelling by primer extension

10-100ng plasmid DNA was boiled for 10min in 20ul H_2O then incubated overnight in a 50ul reaction mix containing, 4ul (5mg/ml BSA), 50uCi 5' [alpha-^{32}P]dGTP or dCTP, 2uM dCTP or dGTP, and 2 units Klenow large fragment of E.coli DNA polymerase in 1x primer extension buffer (see below; Feinberg and Vogelstein, 1983). The labelled DNA was separated from unincorporated triphosphate as described above (19.5).

5x Primer extension buffer was prepared from the following:

1.2x Solution O: 1.5M Tris.HCl pH8, 150mM $MgCl_2$
Solution A: 833ul 1.2x Solution O, 18 ul 2-Mercaptoethanol, 50ul 10mM dATP, 50ul 10mM dTTP, 82ul H_2O
Solution B: 2M Hepes.NaOH pH6.6
Solution C: 50 units $d(N)_6$ in 556ul TE

5x Primer extension buffer = 200A:500B:300C

23. Chemical Sequencing of DNA

The nucleotide sequence of 5' or 3'-end labelled DNA fragments was determined by the chemical methodology of Maxam and Gilbert (1977,1980). Uniquely end labelled DNA restriction fragments were prepared by filling in or phosphorylating a cut RE site then cutting with a second enzyme to produce fragments 50-500bp in length labelled solely on one strand (sections 22.1 and 22.2 above). These were then purified as described above (19.2) and 20-50,000cpm aliquots reacted to modify either purines or cytosine bases. For the purine reaction the DNA was resuspended in 2% Diphenylamine/

1mM EDTA/ 66% formic acid for 2min at 25°C. The DNA was then ether extracted and lyophilized. C residues were modified by treatment with hydrazine in the presence of 1.5M NaCl (2min/ 25°C), stopped with 0.3M NaCl and 0.1M EDTA and ethanol precipitated. The partially modified DNA was then resuspended in fresh 1M piperidine at 90°C for 30min to cleave the DNA at modified bases. The products were precipitated or lyophilized three times and resolved on 8-20% denaturing polyacrylamide gels (19.3).

24. Southern Transfer of DNA to Nitrocellulose

Restricted DNAs for Southern transfer (Southern, 1975) were run on agarose gels in L-buffer. Following electrophoresis the gel was shaken first in Gel Soak I for 1h then in Gel Soak II for 1h. The DNA was blotted overnight onto presoaked Nitrocellulose paper using 10x SSC, a wick of Whatman 3mm paper and a weighted capillary stack of absorbent paper towelling. The filter was then air dried and baked at 80°C for 2h in a vacuum oven prior to hybridization.

25. Immobilization of DNA on Nitrocellulose by Slot-Blot

A Schleicher and Schuell slot-blot apparatus was used to immobilze equivalent quantities of viral infected total cell DNA onto nitrocellulose. DNA was extracted as described above (Section 3B5) and usually digested with RNAase, to remove RNA, and with restriction enzymes to assist accurate pipetting. DNA was denatured with 1vol 1M NaOH (10min/ RT) then neutralized with 2vol 2M ammonium acetate. The samples were then applied to the presoaked nitrocellulose membrane under vacuum using the slot-blot apparatus, and washed through with 1M ammonium acetate. The filter was then air dried and baked at 80°C for 2h in a vacuum oven prior to hybridization.

26. DNA-DNA Hybridization

Nitrocellulose filters were prehybridized in fresh hybridization mix for 2-4h at 65 or 70°C. 10^7-10^8cpm of probe DNA was denatured by addition of 0.04vol 5M NaOH for 10min then neutralized with 5M HCl before adding to the

filter bag and hybridizing overnight under the same conditions as for prehybridization. The filter was then washed twice at RT in 2x SSC for 10min and twice at the hybridization temperature in 2x SSC plus 0.1% SDS for 1h each before autoradiography at -70°C with screens.

27. Colony Hybridization

Transformant E.coli were replica plated onto a master culture dish and a nitrocellulose filter overlain on a plate of selective medium, and grown overnight. The filter was removed from the dish and treated successively with the following solutions by laying colony side down onto each solution on a clean glass plate:

1ml each of	0.5M NaOH	(10min)
	1.0M Tris.HCl pH8	(twice for 1min)
	1.5M NaCl/ 0.5M Tris.HClpH8	(5min)
	chloroform	(1min)

The filters were then dried, soaked in 2xSSC, partially dried then baked for 2h at 80°C under vacuum. The filters were then hybridized as described in Section 2B26 above.

28. In vitro transcription and translation

The pGEM-2 derived plasmids were linearized by restriction enzyme digest before the transcription reaction, and phenol extracted. In vitro transcription was performed using the Riboprobe system (Promega Biotech) following the manufacturer's protocol and incubating 1ug of plasmid DNA, 0.5mM-G(5')ppp(5')G (Pharmacia) and 0.4mM of each nucleoside triphosphate in a total volume of 25ul at 37°C for 1h.

In vitro translation was carried out by addition of 2.5ul of transcription reaction mixture to 20ul of rabbit reticulocyte lysate (Amersham) and incubated for 90min at 30°C in the presence or absence of 25-50 uCi [^{35}S]methionine (>800Ci/mmol) in a 25ul reaction mixture. The radiolabelled translation mixture was processed for SDS-PAGE as described above (Section 2B8) after treatment with 15ul RNAase (200ug/ml) at 30°C for 15min. Non-radioactive samples were used immediately in gel retardation assays (Section 2B15).

29. Computing

Computer analyses of DNA and protein sequences were performed on the PDP 11/14 or MicroVAX II computers in this department. The majority of computing used the University of Wisconsin Genetics Computer Group software package.

CHAPTER 3: RESULTS

SECTION 3A: CONSTRUCTION OF INSERTION AND DELETION MUTANTS WITHIN A PLASMID-BORNE COPY OF THE GENE ENCODING Vmw175.

The aim of the work presented in this thesis was to define the regions of the protein Vmw175 which are important for its various functions and hence to relate the structure of the protein to its biochemical activities. To this end, a large number of in-frame insertion and deletion mutations were introduced into a plasmid-borne copy of the gene encoding Vmw175, and the functional activity of the mutant polypeptides expressed from these plasmids was determined in transiently transfected cells. Thus the regions of the protein sensitive to disruption of function were mapped and related to the primary amino acid sequence of the protein.

1. The plasmid p175

Vmw175 is encoded by IE gene 3 of HSV-1, which is present in two copies on the HSV-1 genome, being carried on the repeat sequences bounding U_S, the short unique region (Rixon et al., 1982, see Figures 2, 3 and 10). The sequence of the gene reveals an uninterrupted reading-frame encoding a protein of 1298 amino acids having a predicted unmodified molecular weight of 133kDa (McGeoch et al., 1986). The coding region of IE gene 3 starts at the ATG at position 1477 in the sequence of the short repeat section of the HSV-1 genome, and continues to the TAA at 5371 (McGeoch et al., 1986). Plasmid p175 (Everett, 1987b) contains the IE gene 3 coding region linked to the SV40 early promoter and enhancer (Figure 10). This construct was chosen to express Vmw175 because the SV40 promoter and enhancer have been shown to respond poorly to HSV-1 IE gene products (O'Hare and Hayward, 1984, 1985a, 1987) and therefore using this construct to express the mutant polypeptides should minimize any complications in the interpretation of the results which would arise from the regulation of the IE 3 promoter by Vmw110 and Vmw175. In this thesis the IE 3 sequence is usually numbered from +1 at the first base of the open reading frame.

2. Construction of insertion and deletion mutations within the Vmw175 coding region

The plasmid p175 was mutagenized by inserting a phosphorylated dodecamer EcoRI oligonucleotide linker (5'-CCCGAATTCGGG-3') at separate blunt-ended restriction sites, essentially as described by Everett (1987b). Plasmid p175 was cut with restriction enzymes (AluI, NaeI, SmaI, BalI or RsaI) in the presence of ethidium bromide in order to produce a maximum of singly cut linear molecules with blunt ends. The precise conditions depended on the enzyme used, but generally 10 ug of plasmid was cut with 10 units of enzyme for 20 minutes at 37°C in the presence of 20-50 ug/ml ethidium bromide in a final volume of 50 ul of the buffer recommended for the enzyme. The DNA was loaded onto a 5-20% sucrose density gradient and centrifuged for 16 h at 28 500 r.p.m. and 15°C in the Sorvall TST41 rotor. Linear DNA sedimented more slowly than circular forms and was easily separated. Linear, phosphatased DNA was directly ligated to blunt ended 12-bp EcoRI phosphorylated linker oligonucleotides. Plasmids with linker inserts were identified by screening small-scale plasmid preparations for the presence of a novel, unique EcoRI site. The site of insertion was first mapped using restriction enzymes and their location and integrity confirmed by DNA sequencing (see Section 3A3 below). The number of linkers inserted was deduced by restriction analysis and, in necessary cases, reduced to a single copy by redigestion with EcoRI, followed by religation.

A summary of the insertion mutants obtained showing their positions and the alterations to the amino acid sequence is given in Figure 11 and Table 4. Thirty-nine inserts were created which span the entire 3.9kb coding region, but, because of the paucity of useful restriction sites in this highly G+C rich gene, the distribution of inserts recovered was uneven.

As a by-product of the insertion mutagenesis several deletions were also recovered and further specified deletions could be created by joining together the coding regions 5' and 3' to the EcoRI sites of two separate insertion mutants. Where appropriate these deletions were

Table 4: Construction of insertion mutants. Plasmid insertion mutants constructed by insertional mutagenesis of pl75 (Section 3A2) are listed in column 1, and the published insertion mutant name recorded in column 3 (Paterson and Everett, 1988b). Column 2 records the coordinate of the first base in the recognition sequence of the restriction site (column 4) at which the 12 bp EcoRI linker has been inserted, numbering from the first base of the IE3 ORF. Disruption of a codon replaces it with five new codons, insertion between codons adds four new codons. Using the single letter code, the amino acid inserted into is listed followed by the five new residues added, or, the two amino acids inserted between are listed followed by the four new residues inserted (column 5). All the plasmid insertion sites have been sequenced, except FN125, which was created by filling in the EcoRI site of deletion plasmid N125 (Table 5).

Table 4: Construction of insertion mutants

1 Construct	2 Site	3 Name	4 Insert site	5 Insertion AA and inserted AA sequence
S38R7	0033	PI1	SMA I	12P-PRIRG
N15	0211	pI2	NAE I	71A/72G-PEFG
A46-4R2	0248	pI3	ALU I	83Q/84L-PEFG
R30	0366	pI4	RSA I	123Y-SRIRD
N113	0388	pI5	NAE I	130A/131G-PEFG
S2	0407	pI6	SMA I	137R-PRIRG
N72	0480	pI7	NAE I	161P-PPNSG
N102	0480	pI7	NAE I	161P-PPNSG
N26	0684	pI8	NAE I	229P-PPNSG
S24R4	0752	pI9	SMA I	252R-PRIRG
N35	0823	pI10	NAE I	275A/276G-PEFG
A117	0875	pI11	ALU I	292E/293L-PEFG
R2A	0927	pI12	RSA I	310Y-SRIRD
S19	0969	pI13	SMA I	324P-PPNSG
R73	0984	pI14	RSA I	329Y-SRIRD
S23R3	1008	pI15	SMA I	337P-PPNSG
R10	1116	pI16	RSA I	373Y-SRIRD
S6R6	1192	pI17	SMA I	398P/399G-PEFG
S48R1	1192	pI17	SMA I	398P/399G-PEFG
N19	1312	pI18	NAE I	438A/439G-PEFG
N78	1480	pI19	NAE I	494A/495G-PEFG
R66	1551	pI20	RSA I	518Y-SRIRD
FN125-1	1681	pI21	NAE I	561A/562G-PELIRG
S4R2	1770	pI22	SMA I	591P-PPNSG
S59-1R2	1770	pI22	SMA I	591P-PPNSG
N123	1945	pI23	NAE I	649A/650G-PEFG
U30R3	2041	pI24	ALU I	681S-SPNSG
S66R5	2319	pI25	SMA I	774P-PPNSG
S41R2	2357	pI26	SMA I	787R-PRIRG
N66	2405	pI27	NAE I	803R-PRIRG
A104	2528	pI28	ALU I	843E/844L-PEFG
A208	2528	pI28	ALU I	843E/844L-PEFG
R74	2799	pI29	RSA I	934Y-SRIRD
N43	2827	pI30	NAE I	943A/944G-PEFG
N77	3061	pI31	NAE I	1021A/1022G-PEFG
S31R1	3061	pI31	NAE I	1021A/1022G-PEFG
N63	3061	pI31	NAE I	1021A/1022G-PEFG
N22	3195	pI32	NAE I	1066P-PPNSG
R34	3291	pI33	RSA I	1098Y-SRIRD
R43	3396	pI34	RSA I	1133Y-SRIRD
N70	3414	pI35	NAE I	1139P-PPNSG
N28	3583	pI36	NAE I	1195A/1196G-PEFG
S95	3690	pI37	SMA I	1231P-PPNSG
N45	3705	pI38	NAE I	1236P-PPNSG
S61	3715	pI39	SMA I	1239P/1240G-PEFG

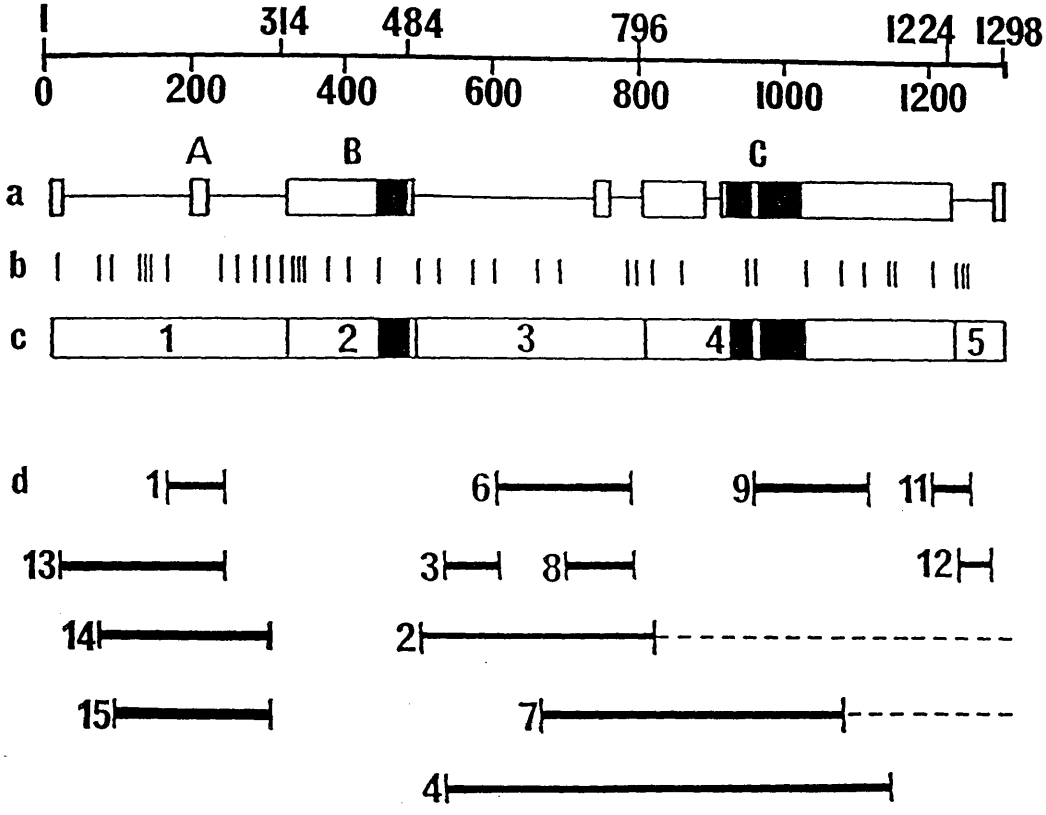


Figure 11: Map of the Vmw175 coding sequence showing the position of the insertion and deletion mutants constructed as described in Section 3A2. (a) Homology map of Vmw175 in relation to VZV 140K. The three main homology regions A, B and C are indicated. Clearly homologous regions are shown as boxes, with the most homologous sections filled (at least 80% identity). (b) Position of each linker insertion (I1-39) is represented by a vertical line. (c) Colinear map showing the 5 regions of Vmw175 as described by McGeoch et al., (1986). (d) Extent of deletions pD1-15. Dotted lines represent out of frame sequences. The scale is given in amino acids.

put back in frame by filling-in or trimming the EcoRI site to achieve the net addition or removal of a base. A summary of the structures of some of the deletion mutants obtained is given in Figure 11 and Table 5.

Small spontaneous deletions were frequently observed to arise in the sequences downstream of IE gene 3 in plasmids derived from pl75. These deletions were probably within the highly repetitive sequences adjacent to and within the a sequences. Restriction analyses confirmed that these deletions did not extend into the IE3 gene (results not shown).

3. Mapping and sequencing the site of mutation

Provisional mapping of the inserted EcoRI sites was performed by restriction analysis of plasmid DNA on 0.8-1.5% agarose gels. Selected plasmids were then prepared on a larger scale and the insertion or deletion sites were mapped more accurately on 3-8% non-denaturing polyacrylamide gels. The number of inserted dodecanucleotide linkers could be calculated from these gels, and plasmids with multiple linker insertions were digested extensively with EcoRI and then religated (plasmids given the "RX" suffix in Table 4).

The site of each inserted EcoRI linker was sequenced by labelling the EcoRI site with dATP and alpha [^{32}P] dTTP and then cutting with a second restriction enzyme to generate uniquely end-labelled fragments 5' and 3' to the inserted linker. After purification on 4-8% polyacrylamide gels the fragments were sequenced by the chemical cleavage protocol of Maxam and Gilbert (1977, 1980). The reaction products were resolved on 20% denaturing polyacrylamide gels, so that sequence ladders (in 5' and 3' directions) from the labelled EcoRI site were generated. These sequences were then compared with the predicted IE gene 3 sequence.

Only two sequencing reactions were performed on each fragment to enable alignment to the known sequence. Purines were modified for cleavage using diphenylamine, and in a separate reaction hydrazine in the presence of high salt was used to preferentially modify cytosine residues; in the event guanine and thymidine were also cleaved to a certain extent which allowed all four bases to be distinguished.

Table 5: Construction of deletion mutants

1 CONSTRUCT NAME	2 PUBLISHED NAME	3 DELETED SEQUENCE	4 DELETED AMINO ACIDS	5 INSERTED AMINO ACIDS	6 FRAME	7 CONSTRUCTION
FA14		36-213	13G-71A	PN-STOP	OUT	S38R7/N15 FILLED IN ECORI
V5	pD13	36-686	12P-229P	PPNSG	IN	S38R7/N26
ZA13	pD14	214-876	72G-292E	PEFG	IN	N15/A117
I11		250-3808	84L-1270A	PEFG	OUT	A46-4R2/L2
Y3	pD15	250-876	84L-292E	PEFG	IN	A46-4R2/A117
D10	pD1	483-686	162A-229P	PNSG	IN	N72/N26
L2		1355-3808	454M-1269L	RIR	IN	BALI DEL./ECO 12MER INSERT
N11	pD2	1484-2407	495G-803R	PEFG	OUT	NAEI DEL./ECO 12MER INSERT
FN11-8	pD2F	1484-2407	495G-803R	PELIRG	IN	N11 FILLED IN ECORI
A3		1553-1772	518Y-591P	SRIR	OUT	R66/S59-1R2
AA2	pD3	1553-1772	518Y-591P	SRINSG	IN	A3 ECO CUT AND FILLED IN
R36	pD4	1553-3397	518Y-1133Y	SRIRD	IN	RSAI DEL./ECO 12MER INSERT
N41		1684-3063	562G-1021A	PEFG	IN	NAEI DEL./ECO 12MER INSERT
N125	pD5	1684-(FS)	562G-(FS)	PEFG	OUT	12MER INTO NAEI/1 BASE DEL.
B4,BA2	pD6	1773-2321	592G-774P	PNSG	IN	S59-1R2/S66R5
E20		1948-2321	650G-774P	PELIR	OUT	N123/S66R5 FILLED IN ECORI
N59	pD7	1948-3197	650G-1066P	PEFG	OUT	NAEI DEL./ECO 12MER INSERT
FN59-4	pD7F	1948-3197	650G-1066P	PELQACNSO	IN	N59 ECO FILLED + HIND 10MER
G3		2043-2321	682S-774P	PNCKLAIR	OUT	U30/S66R5 ECO FILL + HIND 10MER
GA6	pD8	2043-2321	682S-774P	PNSP	IN	U30R3/S66R5
R9	pD9	2801-3292	934Y-1098Y	SRIRD	IN	RSAI DEL./ECO 12MER INSERT
C1,CA7	pD11	3586-3717	1196G-1239P	PEFG	IN	N28/S61
H1,H6	pD12	3693-3808	1232G-1269L	PNSKLAIR	IN	S95/L2 ECO FILL + HIND 10MER

Table 5: Construction of deletion mutants

Columns 1 and 2 list deletion derivatives of plasmid pl75 giving both their construction name and simplified published name (pD series, Paterson and Everett, 1988a,b). The extent of the deletion is defined both in bases (relative to +1 at the start of the IE3 ORF) and amino acids (columns 3 and 4). The deletion end points were confirmed by sequencing except in the cases of pD13, pD14 and pD15. Column 4 also records the amino acid deleted at each deletion boundary; column 5 records the novel amino acids inserted at the deletion site. Column 6 records whether the reading frame is correctly reinitiated after the deletion. Column 7 records the construction protocol for each plasmid; deletion plasmids recovered spontaneously from insertion mutagenesis are described as DEL./ECO 12MER INSERT; for plasmids constructed by in vitro recombination the 5' then 3' parents are listed. Where necessary the reading frames were adjusted by filling in the EcoRI site (with DNA polymerase Klenow fragment) and in some cases ligating a 10 base pair HindIII linker into the filled site.

Figure 12 shows a representative sequence analysis for four separate insertion mutants: S19, S48, R2A and R30.

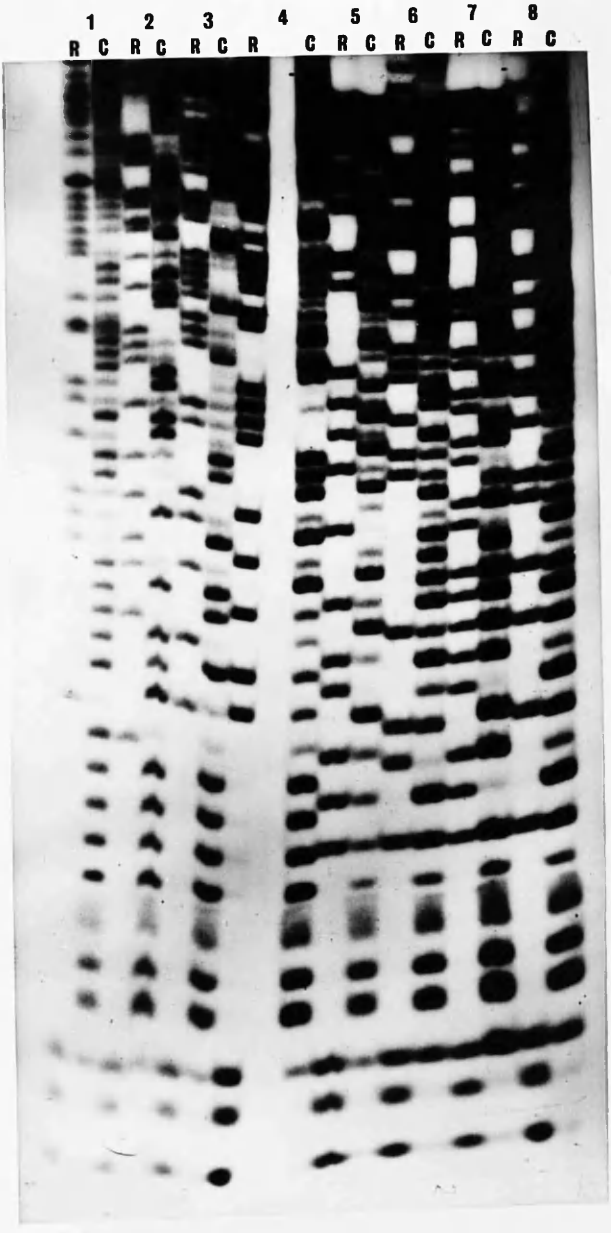
4. Construction of pGEM175: a vector for in vitro expression of Vmw175.

Reports that purified Vmw175 is capable of binding to DNA (eg Michael et al., 1988) suggested the possibility that Vmw175 expressed in vitro might also be capable of binding to DNA in the gel retardation assay. The recent success of Ace et al., (1988) in expressing the HSV-1 alpha-TIF, Vmw65, in vitro from a T7/SP6 transcription vector, pGEM-2, provided a hopeful protocol to exploit. Because the plasmid pGEMTIF can be used to express adequate amounts of Vmw65 in vitro (Ace et al., 1988) it was decided to use this plasmid to create a fusion between the first five amino acids of Vmw65 and a large portion of Vmw175, rather than attempt to achieve efficient expression from a de novo construct. The construction of pGEM175 is outlined in Figure 13a. The insertion of the PvuII-HincII fragment from p175 into the SalI deleted pGEMTIF (pdelS2) creates a gene encoding the first five amino acids of Vmw65, fused in frame to residues 84-1029 of Vmw175, terminating in 64 residues encoded by the out of frame 3' end of the Vmw65 gene. The desired recombinant was detected by colony hybridization of ampicillin resistant transformants as described in the Materials and Methods and using a probe internal to the IE3 coding sequence, labelled by primer extension. Figure 13b shows the autoradiograph obtained from this screening, with the colonies of pGEM175 and pGEM571 arrowed. (pGEM571 contains the PvuII-HincII fragment in the reverse orientation, and was used as a control in some experiments.)

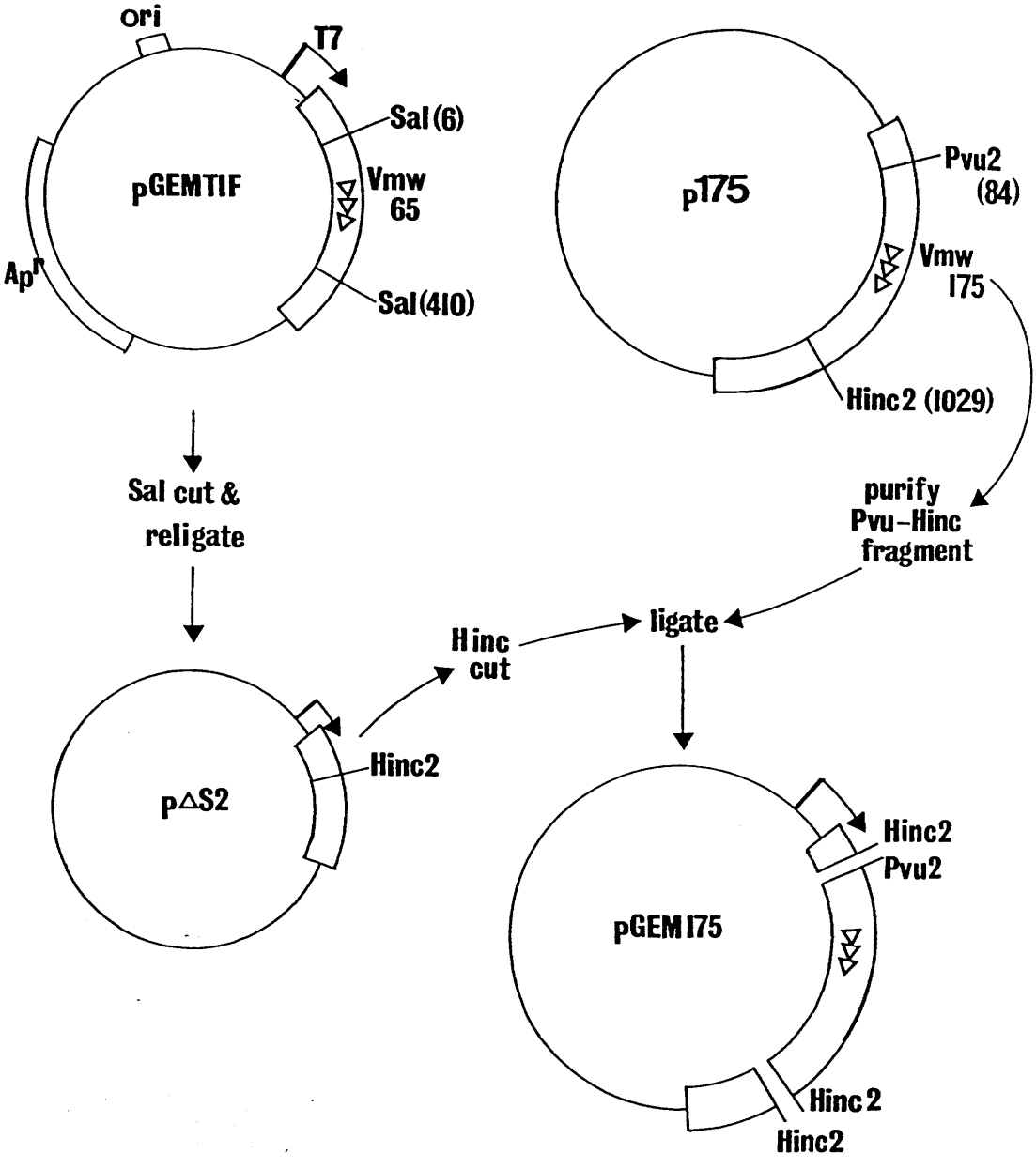
Figure 12: Representative sequence analyses of four insertion mutants. Fragments 3' and 5' to the site of insertion, uniquely end labelled at the EcoRI site were prepared as described in the text and in Materials and Methods. Each fragment was treated to cleave at purines (lane R) or preferentially at cytosines (lane C) and resolved on a 20% denaturing polyacrylamide gel and autoradiographed at -70°C with intensifying screen. Lanes: (1) S19-3' (2) S19-5' (3) S48-5' (4) S48-3' (5) R2A-3' (6) R2A-5' (7) R30-3' (8) R30-5'

Sequences read:
(1) AAGCCC-CCCCCGCCCCCGGGCCGCGGCCC
(2) AAGCCC-CCCCGCCCACGACATGCCGCC
(3) AAGCCC-CCCTGCACCGCGACCTGGT
(4) AAGCCC-CCCCTGGTGCGCGCCCAAGA
(5) AAGCCC-TGGGCAGCGCTATCGCGCGCA
(6) AAGCCC-TGCAGTCGCCCCTCGGCACCGGG
(7) AAGCCC-TGAGCAGGGGCAGTAGCAGCAG
(8) AAGCCC-TGCTGCTGCGTCGGCTGCGGCGG

The first base read on each sequencing ladder is the third base of the fragment, adjacent to the two labelled T residues, and reads AAGCCC from the inserted EcoRI linker before entering IE3 sequences.


1 2 3 4 5 6 7 8
R C R C R C R C R C R C R C

A


Ori
T7
Sal(6)
pGEMTIF
Vmw
65
Ap^r
Sal(410)
p175
Pvu2
(84)
Vmw
175
Hinc2 (1029)
Sal cut &
religate
purify
Pvu-Hinc
fragment
Hinc
cut
ligate
pΔS2
Hinc2
Hinc2
Pvu2
pGEM175
Hinc2
Hinc2

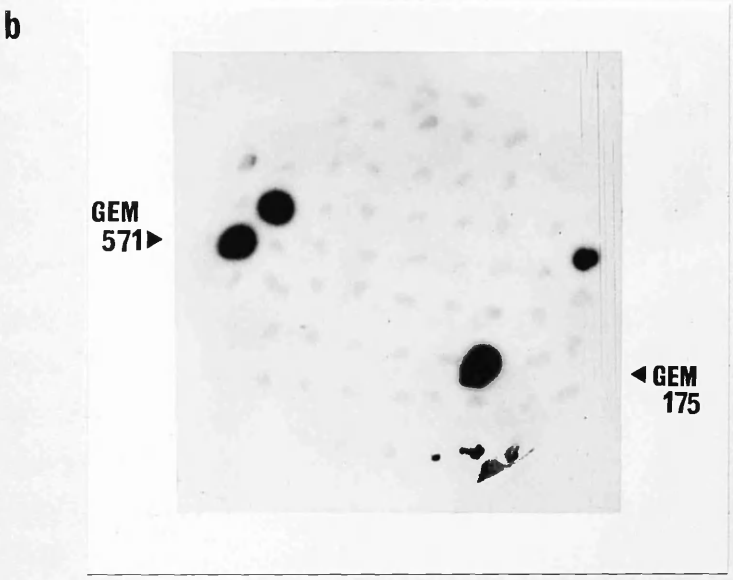


Figure 13: Construction of pGEM175. (a) pGEM175 was constructed from pGEMTIF (Ace et al., 1988) which carries the gene expressing the HSV-1 alpha-TIF, Vmw65, under a prokaryotic coliphage T7 promoter in the vector pGEM-2. The portion of the gene encoding amino acids 6-410 was deleted from pGEMTIF by SalI digestion and religation, to create the plasmid pdelS2. The purified 2.8kb PvuII-HincII fragment from IE gene 3 (from pl75) was then ligated into pdelS2 linearized with HincII. The novel fusion reading frame encoded the first 5 amino acids of Vmw65, fused in frame to residues 84-1029 of Vmwl75, and terminates in an out of frame section of the Vmw65 reading frame which terminates after 64 amino acids. (b) Autoradiograph of colony hybridization experiment to detect the pGEM175 recombinant. DNA from ampicillin resistant transformants was probed with a primer extended PvuII-EcoRI fragment from pI31 (bases 246-3061 of the IE3 ORF). Positive colonies for pGEM175 and pGEM571 (with the PvuII-HincII fragment cloned in the reverse orientation) are shown.

SECTION 3B: VISUALIZATION OF THE WILD-TYPE AND MUTANT Vmw175 POLYPEPTIDES IN TRANSFECTED HELA CELLS

Indirect immunofluorescence studies were performed on cells transfected with wild-type and mutant plasmids in order to visualize the production and localization of Vmw175. It was originally hoped to use an antiserum which recognizes the carboxyl terminus of Vmw175 in order to confirm that mutant plasmids produce proteins which terminate in the correct reading frame. However, rabbit antisera directed against a synthetic oligopeptide corresponding to the carboxyl terminus of Vmw175 failed to recognize Vmw175 in either infected or transfected cells in this assay (results not presented). We therefore used the monoclonal antibody 58S (Showalter et al., 1981) which probably recognizes an epitope near the carboxy terminus (see below).

Formaldehyde-fixed, transfected HeLa cells were incubated with the antibody and then with fluorescein-conjugated swine anti-mouse Ig, as described in Materials and Methods (Section 2B12). Figure 14a shows cells transfected with pUC9 alone, giving no positive fluorescence. Figures 14b and 14c show cells transfected with p175; 5-20% of the cells showed strong nuclear fluorescence indicating the accumulation of Vmw175 in the nucleus. All of the insertion mutants listed in Table 4 gave rise to similar nuclear fluorescence. These results indicate that there are unlikely to be any secondary unselected mutations in the plasmid-borne mutants.

The immunofluorescence results for the deletion mutants are shown in Table 6. The out of frame deletions fail to give rise to immunofluorescence suggesting that the epitope recognized by 58S has been lost, or that these mutants do not accumulate in sufficient quantities to allow detection. Considering the extent of the in frame deletions (which all give rise to proteins recognized by 58S) the results indicate that the epitope recognized by this antibody lies towards the C-terminus of the protein, between residues 1134-1195 or 1270-1298. Some deletions gave rise to disperse cytoplasmic immunofluorescence (Figures 14d and

14e). These results indicate that the signal required for nuclear localization, or at least an essential portion of it, lies between amino acids 682-774 (mutant pD8). The limits of this signal have not been defined in this study, nor can it be excluded that there are other signals in regions of the protein that have not been deleted. However, DeLuca and Schaffer (1988) have also mapped the nuclear localization signal to between residues 590 and 773 using truncation mutants (see Section 1D2.4). This region of Vmw175 contains a short basic amino acid string RKRKS at residue 727, which is conserved in the VZV 140K protein and resembles the nuclear localization signal characterized in SV40 T antigen (Kalderon et al., 1984; see Section 4.3.3).

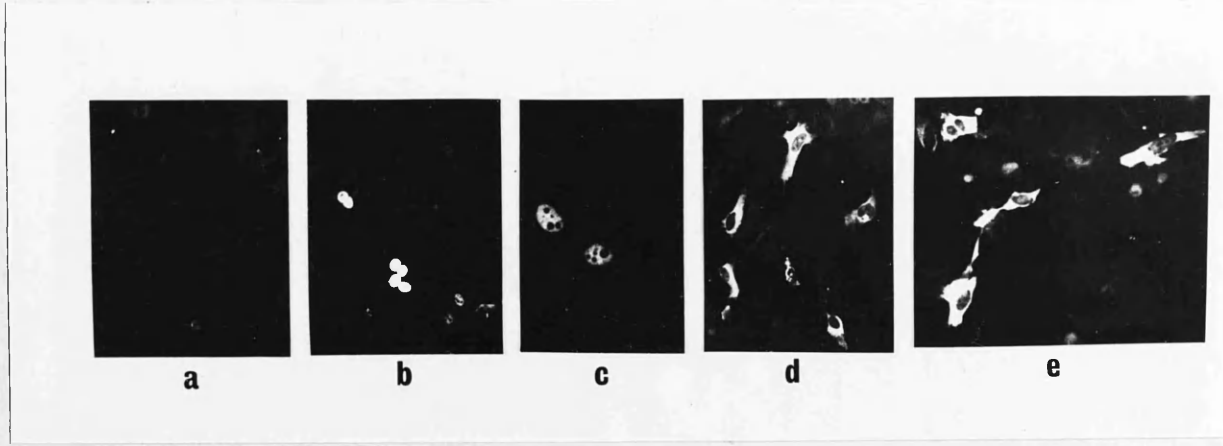


Figure 14: Immunofluorescence of transfected HeLa cells stained with the monoclonal antibody 58S. WSHeLa cells on 50mm plastic Petri dishes were transfected with 10ug of each plasmid and then fixed and stained with the anti-Vmw175 monoclonal antibody 58S as described in Materials and Methods (Section 2B12). (a) Mock-transfected cells. (b and c) cells transfected with p175 showing intense nuclear staining (b at X75 and c at X300 magnification). (d) Cells transfected with pD2F and (e) with pD8 both exhibiting cytoplasmic localization of the proteins made by these mutants.

Table 6: Immunofluorescence of transfected Hela cells with monoclonal antibody 58S.

1 CONST. NAME	2 PUBL. NAME	3 DELETED SEQUENCE	4 DELETED AMINO ACIDS	5 FRAME	6 I.I.F.
FA14		36-213	13G-71A	OUT	-ive.
V5	pD13	36-686	12P-229P	IN	N.D.
ZA13	pD14	214-876	72G-292E	IN	N.D.
I11		250-3808	84L-1270A	OUT	-ive.
Y3	pD15	250-876	84L-292E	IN	N.D.
D10	pD1	483-686	162A-229P	IN	nuc.
L2		1355-3808	454M-1269L	IN	-ive.
N11	pD2	1484-2407	495G-803R	OUT	-ive.
FN11-8	pD2F	1484-2407	495G-803R	IN	cyt.
A3		1553-1772	518Y-591P	OUT	-ive.
AA2	pD3	1553-1772	518Y-591P	IN	nuc.
R36	pD4	1553-3397	518Y-1133Y	IN	cyt.
N41		1684-3063	562G-1021A	IN	-ive.
N125	pD5	1684-(FS)	562G-(FS)	OUT	-ive.
B4,BA2	pD6	1773-2321	592G-774P	IN	cyt.
E20		1948-2321	650G-774P	OUT	-ive.
N59	pD7	1948-3197	650G-1066P	OUT	-ive.
FN59-4	pD7F	1948-3197	650G-1066P	IN	cyt.
G3		2043-2321	682S-774P	OUT	N.D.
GA6	pD8	2043-2321	682S-774P	IN	cyt.
R9	pD9	2801-3292	934Y-1098Y	IN	nuc.
C1,CA7	pD11	3586-3717	1196G-1239P	IN	nuc.
H1,H6	pD12	3693-3808	1232G-1269L	IN	nuc.

Table 6: Immunofluorescence of transfected Hela cells with monoclonal antibody 58S. Columns 1-5 are reproduced from Table 5. The cellular localization of each deleted polypeptide as determined by indirect immunofluorescent staining (IIF) of transfected cells is recorded in column 6. Formaldehyde-fixed, transfected HeLa cells were incubated with the antibody 58S and then with fluorescein-conjugated swine anti-mouse Ig, as described in Materials and Methods (Section 2B12). (N.D. = not done, -ive = no detectable immunofluorescence, nuc. = nuclear immunofluorescence similar to wild-type Vmw175, cyt. = cytoplasmic immunofluorescence as in Figure 14). Columns 1 and 2 list deletion derivatives of plasmid p175 giving both their construction name and simplified published name (pD series, Paterson and Everett, 1988a,b). The extent of the deletion is defined both in bases (relative to +1 at the start of the IE3 ORF) and amino acids (columns 3 and 4). Column 5 records whether the reading frame is correctly reinitiated after the deletion.

SECTION 3C: DETERMINATION OF THE TRANSCRIPTIONAL REGULATORY ACTIVITY OF THE PLASMID-BORNE MUTANTS OF IE GENE 3.

The primary aim of the work presented in this thesis was to map the regions of Vmw175 that are functionally important by using the in-frame insertion and deletion mutants whose construction is described above (Section 3A) to define which areas of the protein are sensitive to disruption of activity. In order to assay the transcriptional activity of the mutant polypeptides, transient transfection assays were performed in which the ability of each mutant protein to activate or repress a reporter gene was measured. Two reporter gene constructs were used, both being fusions of HSV-1 promoters to the bacterial chloramphenicol acetyl transferase gene. One plasmid construct, pgDCAT, contains the promoter from the HSV-1 early gene which encodes glycoprotein D, and was used to measure transactivation of expression by Vmw175 in synergy with the other HSV-1 promiscuous transactivator Vmw110; the other reporter plasmid, pIE3CAT, carried a fusion of the HSV-1 IE gene 3 promoter to the CAT reading frame, and was therefore used to measure autoregulation of the promoter of the viral gene which encodes Vmw175.

Transfections were performed on WS HeLa or Vero cells seeded at $1x10^6$ cells per 50mm plate as described in Materials and Methods (Section 2B9.1) and the CAT enzyme activity determined on extracts made 48h post transfection (Section 2B10). The percentage conversion of substrate to acetylated form was calculated per microgram of protein in the extract (Section 2B11) and the CAT activities compared between wild-type and mutant Vmw175 transfections.

1. Assay of transactivation of the HSV-1 glycoprotein gD promoter by Vmw175, and the insertion mutants.

It has been shown that the cloned IE-1 and IE-3 gene products from the HSV-1 Glasgow strain 17 syn^+ stimulate the HSV-1 gD promoter synergistically in short-term transfection experiments in HeLa cells (Everett, 1984). This assay was used to examine the phenotype of the insertion and deletion mutants in Vmw175. In these experiments, the gD promoter

(from residues -392 to +11) was linked to the chloramphenicol acetyl transferase (CAT) gene in the plasmid pgDCAT (Everett, 1986) and Vmw110 was provided by the plasmid p111 (Everett, 1987b). Transfections were performed with 4ug pgDCAT and p111, and 8ug of the plasmid expressing Vmw175. Under these conditions the amount of p175 was near the top of the dose response curve in order to minimize any fluctuations in activity due to errors in DNA quantitation (see Figure 15). Typical results using this assay are shown in Figure 16a. When HeLa cells were transfected with pgDCAT alone, the resultant cell extracts contained only low levels of CAT activity (track 1). This low level of activity was increased about 3- to 5-fold when either p175 or p111 were included in the transfection with pgDCAT (tracks 2 and 3 respectively). However, when both p111 and p175 were present in the transfection, the activity obtained from pgDCAT was on average almost 20 times that obtained with activation by p111 alone (track 4). This 20-fold increase was used as the assay to test the transactivation phenotypes of the insertion and deletion mutants in the Vmw175-coding region of p175. It has previously been demonstrated that results from similar CAT assays correlate closely with the transcription levels as measured by direct quantitation of cytoplasmically accumulated RNA (Everett, 1987b).

The 39 insertion mutants in Vmw175 shown in Figure 11 and Table 4 were assayed as described above. To measure transactivation of the gD promoter in the presence of Vmw110 at least two independent preparations of each plasmid DNA were used for a total of at least five transfection experiments. In each transfection experiment, two positive control plates with p175 were prepared and the average activity (percentage chloramphenicol acetylated per mg protein in a 30 minute incubation) from these two plates was used to calculate the relative activities of the mutants. The results are given in Table 7, together with the standard errors of the means of the individual values, and the data are plotted graphically in Figure 17. Activation of pgDCAT by p111 alone gave an average activity 6% of that with both p111 and p175, and this background level has not been subtracted from the results shown.

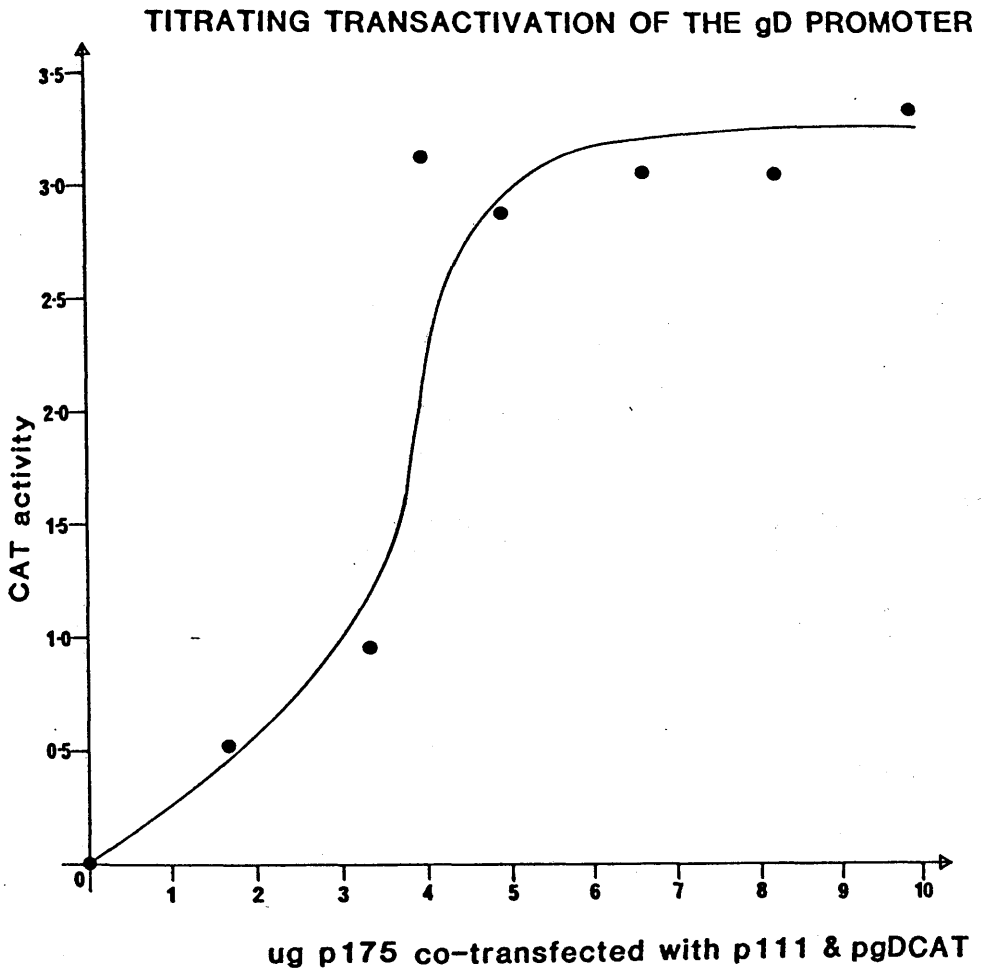


Figure 15: Titrating transactivation of the gD promoter by p175. WSHeLa cells were cotransfected with increasing amounts of p175 and 4ug each of p111 and pgDCAT. CAT extracts were prepared and assayed as described in the Materials and Methods and the CAT activity determined as percentage conversion of substrate to (3)-acetylated product per ug protein in the extract. The results are plotted and show that maximal transactivation of pgDCAT requires addition of around 6-8ug p175.

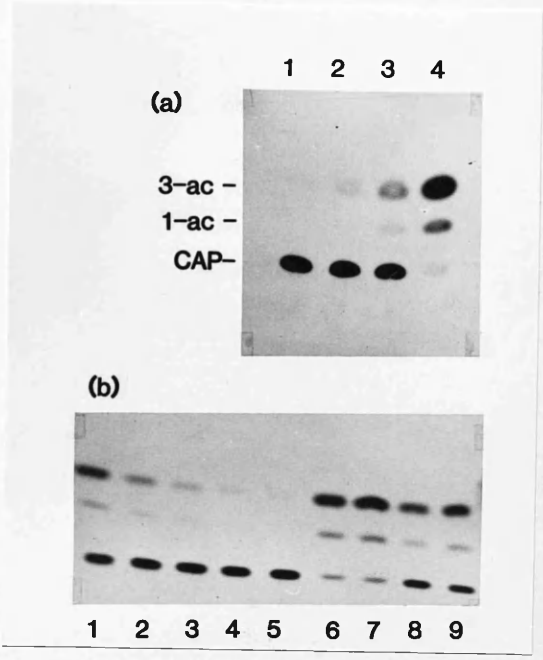


Figure 16: Autoradiograph of CAT assays showing the two phenotypes of Vmw175 being investigated. (a) WS HeLa cells transfected with pgDCAT alone (track 1), pgDCAT with either p175 or p111 respectively (tracks 2 and 3) and pgDCAT, p175 and p111 (track 4). The results show that Vmw110 or Vmw175 alone transactivate CAT expression around 5-fold, whilst in synergy they activate expression a further 20-fold. (b) Transfection of Vero cells with pIE3CAT alone (track 1) or with the addition of increasing amounts of p175 (tracks 2-5; 1,2,4 and 8 ug) or pSVEB (tracks 6-9; 0.5,1,2 and 4 ug); these results are plotted in Figure 18 as specific activities, taking account of the protein concentrations of the extracts. Cotransfection of increasing amounts of p175 cause repression of the IE3 promoter which is not caused by cotransfection of increasing amounts of the promoter alone, cloned on pSVEB.

Table 7: The transactivation and repression activities of the insertion mutants. The activity of each of the 39 insertion mutants pI1-pI39 is recorded for each of the two transfection assays measuring transactivation and autoregulation. The insertion site in each mutant is given in bases (column 2) and amino acids (3). Column 4 records the ability of each mutant to transactivate the viral early promoter, gD, when cotransfected with the reporter plasmid pgDCAT and the plasmid pl11 which expresses Vmwl10. The activity is given as a percentage of that obtained in parallel experiments with pl75. The mean (x) of at least five independent determinations, using at least two separate plasmid preparations, is given, with the standard error of the mean (SEM). Column 5 records the qualitative ability each mutant polypeptide expressed by the plasmids pI1-pI39 to repress the IE3 promoter of pIE3CAT. Repression activity was determined relative to wild-type Vmwl75 expressed from pl75 by titrating repression of the IE3CAT construct and plotting each experiment as in Figure 18. Repression activity is scored from - (essentially no activity) to +++ (wild-type activity).

Table 7: The transacivation and repression activities of the insertion mutants.

1 Mutant	2 Site (base)	3 Site (amino acid)	4 Transactivation activity x (SEM)	5 Repression activity
pI1	0033	12	35.1 (11.7)	+++
pI2	0211	71/72	53.4 (13)	++
pI3	0248	83/84	49.6 (6.7)	++
pI4	0366	123	65.6 (12.8)	++
pI5	0388	130/131	43.8 (6.5)	+++
pI6	0407	137	54.4 (11.8)	++
pI7	0480	161	44.5 (10.5)	+++
pI8	0684	229	45.7 (8)	+++
pI9	0752	252	127 (43.2)	+++
pI10	0823	275/276	25.8 (4.6)	-
pI11	0875	292/293	30.8 (6.3)	++
pI12	0927	310	20.0 (4.4)	+
pI13	0969	324	10.0 (2.9)	-
pI14	0984	329	4.9 (0.9)	-
pI15	1008	337	4.5 (0.9)	+
pI16	1116	373	16.5 (2.8)	-
pI17	1192	398/399	11.8 (1.9)	-
pI18	1312	438/439	15.5 (4.4)	-
pI19	1480	494/495	49.2 (10.4)	+++
pI20	1551	518Y	47.2 (10.2)	++
pI21	1681	561/562	43.4 (4.2)	+++
pI22	1770	591	26.2 (6.2)	++
pI23	1945	649/650	23.9 (5.6)	++
pI24	2041	681	21.1 (4.2)	++
pI25	2319	774	38.2 (6.1)	+++
pI26	2357	787	80.9 (18.5)	+++
pI27	2405	803	45.9 (9.6)	++
pI28	2528	843/844	10.8 (3.9)	+++
pI29	2799	934	3.4 (0.5)	+
pI30	2827	943/944	48.2 (12.3)	++
pI31	3061	1021/1022	22.4 (3.7)	+++
pI32	3195	1066	10.3 (3.0)	+++
pI33	3291	1098	30.0 (8.9)	++
pI34	3396	1133	20.8 (2.3)	++
pI35	3414	1139	48.2 (13.6)	++
pI36	3583	1195/1196	86.9 (18)	+++
pI37	3690	1231	47.5 (10.3)	+++
pI38	3705	1236	63.1 (11.2)	+++
pI39	3715	1239/1240	51.1 (8.6)	+++

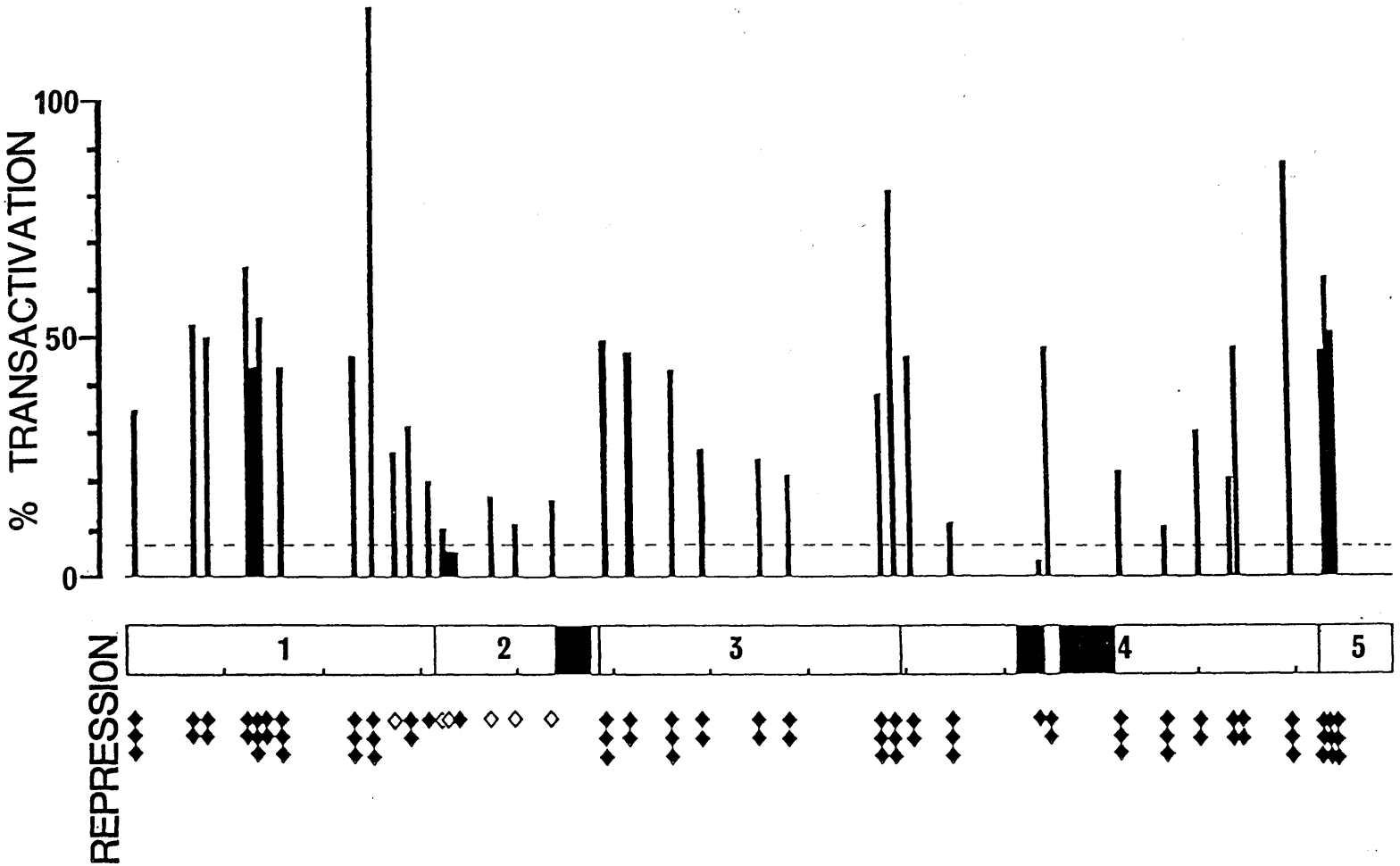

100
50
0
% TRANSACTIVATION
1
2
3
4
5
REPRESSION

Figure 17: Summary diagram showing the relative transactivation and repression activities of the insertion mutants compared to wild-type. The map of the gene with the five structural regions is drawn as described in Figure 11. The transactivation values of each insert are plotted above their site of insertion. The values are taken from Table 7 and show transactivation of pgDCAT in synergy with Vmw110 relative to wild type. The dotted line shows the background level of activity achieved with pgDCAT and Vmw110 (from p111) alone. The qualitative repression activity of each mutant relative to wild-type is represented below the site of insertion. These are reproduced from Table 7 using three diamonds to represent good activity (+++), two: fair (++), one: fairly poor (+), and an empty diamond representing poor or essentially no repression activity (-).

Somewhat unexpectedly all except one of the mutants have lower transactivation activities than pl75, indicating that the protein is sensitive to the insertion of four residues in many regions. The inserted amino acids could affect the packing of the polypeptide chain in a number of ways and it is probably significant that all of the inserts introduce proline or charged residues (see Table 4). However the large class of mutants with 40-50% activity are still strong activators, showing 6-8 fold stimulation over the effect of pl11 alone. There are clearly mutants that are more seriously impaired than this, to 20-30% activity or to virtually no activity at all. There appears to be no obvious correlation between the sequence of novel amino acids introduced, given in Table 4, and the phenotype of the mutants. However, we cannot discount the possibility that the nature of the residues inserted may be more disruptive at some sites of insertion than others, if, for example, a srong charge is introduced into an uncharged region.

All the inserts within the first 230 amino acids of region 1 have between 35% and 65% activity. Therefore this large block seems relatively unimportant for this function although it does show some conservation with the VZV 140K protein, (region A in Figure 11). The insert at amino acid 252, pI9, apparently has greater than wild type activity, though an increase of only 1.25 fold is probably not significant in this assay. The section of Vmwl75 spanning the end of region 1 and all of region 2 is clearly the largest stretch of the protein sensitive to disruption and must represent a critically important domain for functional activity. The three inserts around amino acid 330 are particularly impaired. Region 3 of Vmwl75 seems relatively unimportant in this assay. However, the three central mutations in region 3 are significantly reduced in activity to 20-30% that of pl75. Areas within region 4 are also sensitive to disruption and therefore probably important functionally for transactivation. Interestingly, the mutant pI29, which falls in a region very highly conserved between HSV-1 and VZV, is greatly impaired whilst the nearby insert pI30 functions well in this assay and lies between two highly conserved blocks. The inserts towards the end of

region 4 and the three at the start of region 5 would appear to define a region of the protein unimportant in this assay.

2. Assay of repression of the HSV-1 IE gene 3 promoter by Vmw175, and the insertion mutants.

It has been shown that cloned Vmw175 can repress both basal and trans- and cis-activated levels of transcription from the IE gene 3 promoter (O'Hare and Hayward, 1985b, 1987). Using the same cell-type in which the original studies were performed, Vero cells, it has been possible to reproduce the results of O'Hare and Hayward (1985b) who demonstrated repression of the Vmw110-transactivated IE-3 promoter by adding increasing amounts of Vmw175. To assay this phenotype in short-term transfection experiments we used a plasmid (pIE3CAT) carrying a fusion of the CAT gene to the intact IE-3 promoter, from bases -331 to +26 relative to the cap site (Stow et al., 1986). However, this assay occasionally gave problems of reproducibility and artificial repression possibly due to promoter competition, and these results are not presented here. These problems were overcome by titrating repression on the basal level of expression from pIE3CAT. Figure 16b shows a typical assay using increasing amounts of p175 to supply Vmw175 compared to the negative control, pSVEB, which carries the same promoter fragment but no coding sequences. Clearly under these conditions the repression is dependent on the presence of functional Vmw175. The titrations plotted in Figure 18 demonstrate the absence of promoter competition by pSVEB and the failure of two insertion mutations, pI13 and pI17, to repress.

This assay was used to titrate the repression activity of all the mutants. The results of each set of transfections were plotted as in Figure 18 to obtain a qualitative comparison of the repression with that of the positive control p175. The results are recorded in Table 7 and Figure 17. Plasmids able to repress pIE3CAT essentially as well as p175 are given +++ (or three diamonds in Figure 17). Plasmids which were clearly impaired in their activity but still achieved a significant repression are described with ++; plasmids which repressed barely more effectively

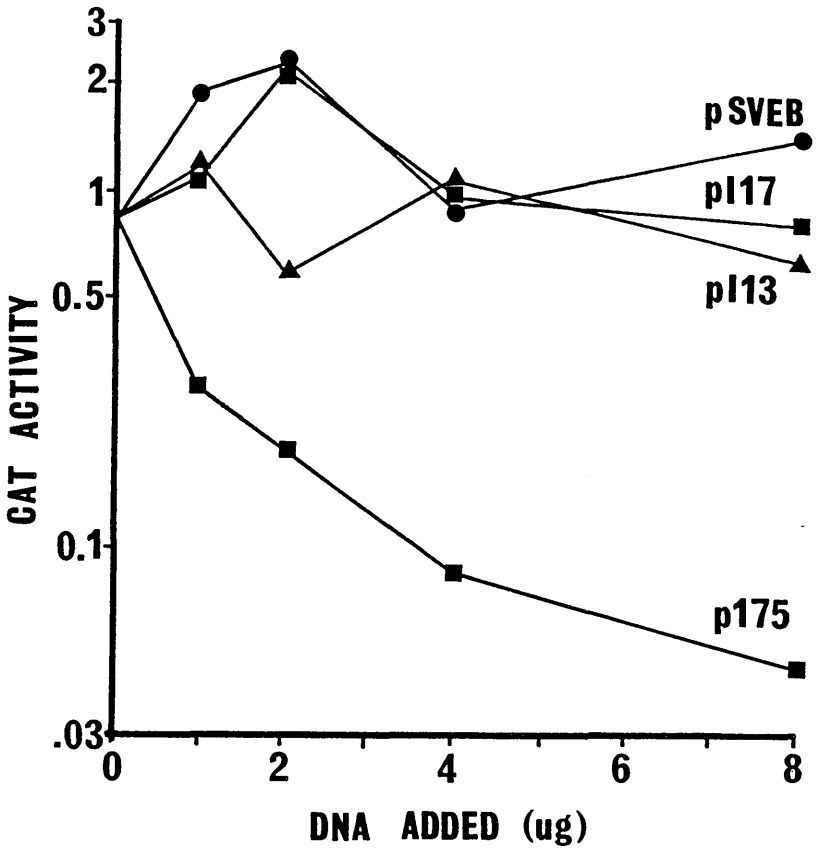


Figure 18: Graph showing the repression of pIE3CAT by pl75. The CAT activity per protein obtained from transfected Vero cells is shown plotted against amount of test plasmid added. 4 ug of pIE3CAT was added in each case and the total amount of DNA added balanced with pUC9. Actual amounts of pSVEB were 0.5,1,2 and 4 ug to provide a similar molarity to the larger pl75 plasmid. pl75 achieves good repression in contrast to a plasmid lacking IE 3 coding sequences, pSVEB. Two mutant plasmids, pIl3 and pIl7, fail to repress (see Table 7). The results demonstrate that the repression is specific to functional Vmwl75 protein and not the DNA sequences in pl75.

than pSVEB are given as + and plasmids with no detectable repression activity are recorded as - (an empty diamond in Figure 17).

The results from these repression assays are probably less sensitive than the transactivation results, being qualitative rather than quantitative. The general areas of Vmw175 sensitive for disruption of the repression phenotype overlap with those defined above for transactivation. However, individual inserts show that the two phenotypes are separable in some cases and are not determined by exactly the same protein regions. Region 2 and the downstream end of region 1 are highly sensitive to disruption, as in the transactivation assay above, but the section defined by inserts at the end of region 1, in particular pI10, seems to be more important for repression than transactivation. The rest of region 1 again seems unimportant for repression, as do regions 3 and 5. Most of the inserts in region 4 are efficient repressors; therefore this region seems to be less important for repression than for transactivation. Mutant pI29, however, is greatly impaired for both functions which suggests that the very highly conserved area (region C in Figure 11) is important for both functions. Therefore, apart from the sequence disrupted by the insertion in pI29, region 1 from amino acid 275 together with region 2 is the only part of Vmw175 we have defined that is critically sensitive to disruption of the repression phenotype. It is possible, however, since the mutational dissection is not exhaustive, that important regions have been overlooked because of the random distribution of insertion sites.

3. Analysis of the transactivation and repression activity of the deletion mutants in Vmw175.

A number of deletion mutants recovered fortuitously from the insertion mutagenesis, or constructed by recombining two insertions (described in Table 5), were assayed for both repression and transactivation activity as described above. The results are summarized in Table 8 and the extents of the deletions are shown in Figure 11.

The results are rather more complicated than those obtained with the insertion mutants. The deletion pD1 in

Table 8: The transactivation and repression activities of deletion mutants.

1 Mut.	2 Deleted bases	3 Deleted AA	4 Frame	5 IIF	6 Transact. activity x (SEM)	7 Repress. activity
pD1	483-686	162-229	IN	nuc.	30 (4.8)	+++
pD2	1483-2407	495-803	OUT	-	23 (4.0)	-
pD2F	1483-2407	496-803	IN	cyt.	5 (0.5)	+
pD3	1553-1772	518-591	IN	nuc.	4 (0.5)	+++
pD4	1553-3397	518-1133	IN	cyt.	18 (3.3)	++
pD5	1684	562	OUT	-	3 (1.3)	++
pD6	1773-2321	592-774	IN	cyt.	29 (2.8)	+
pD7	1948-3197	650-1066	OUT	-	27 (5.4)	-
pD7F	1948-3197	650-1066	IN	cyt.	10 (1.5)	+
pD8	2043-2321	682-774	IN	cyt.	57 (5.6)	-
pD9	2801-3292	934-1098	IN	nuc.	4 (0.6)	+++
pD11	3586-3717	1196-1239	IN	nuc.	6 (2.2)	+++
pD12	3693-3808	1232-1269	IN	nuc.	7 (2.2)	++

Table 8: The transactivation and repression activities of deletion mutants. The activity of each of the 13 deletion mutants pD1-pD12 is recorded for each of the two transfection assays measuring transactivation and autoregulation. The extent of the deletion in each mutant is given in bases (column 2) and amino acids (3). Column 6 records the ability of each mutant to transactivate the viral early promoter, gD, when cotransfected with the reporter plasmid pgDCAT and the plasmid p111 which expresses Vmw110. The activity is given as a percentage of that obtained in parallel experiments with p175. The mean (x) of at least five independent determinations, using at least two separate plasmid preparations, is given, with the standard error of the mean (SEM). Column 7 records the qualitative ability each mutant polypeptide expressed by the plasmids pD1-pD12 to repress the IE3 promoter of pIE3CAT. Repression activity was determined relative to wild-type Vmw175 expressed from p175 by titrating repression of the IE3CAT construct and plotting each experiment as in Figure 18. Repression activity is scored from - (essentially no activity) to +++ (wild-type activity). The reading frame of each mutant 3' to the deletion is also recorded (4) and the immunofluorescence results are reproduced from Table 6: (nuc. nuclear localization similar to p175, cyt. cytoplasmic localization, - no detectable immunofluorescence.)

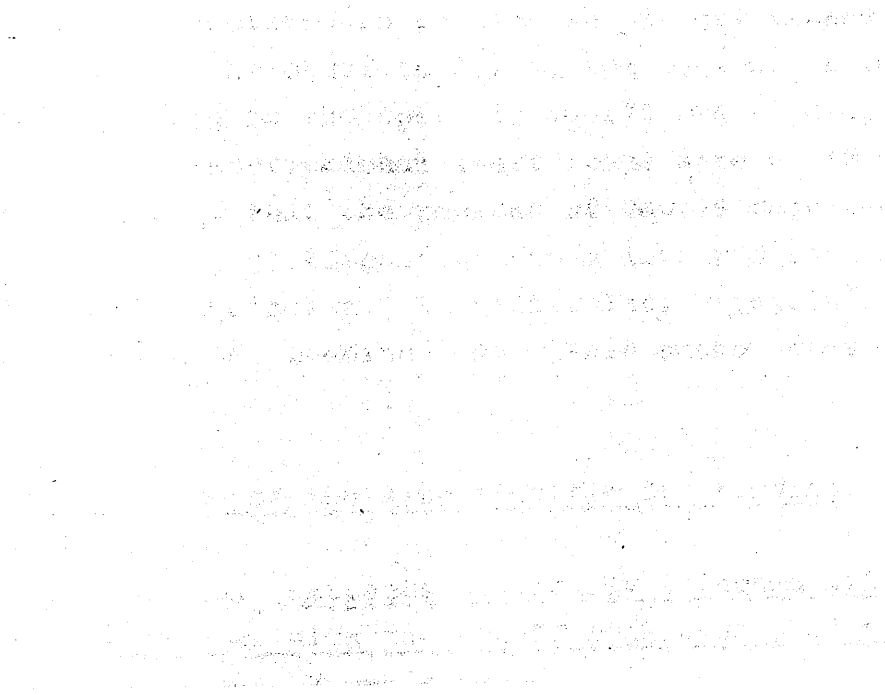
region 1, which removes amino acids 162-229, including the conserved serine rich tract (see Section 4.3.1), has good transactivating and repression activity. Although the results obtained with the insertion mutants suggested that much of region 1 is unimportant for transcriptional regulation, it was expected that the deletion of an evolutionarily preserved motif would have a marked effect on activity. This serine-rich region has been proposed as the major phosphorylation site of Vmw175 (DeLuca and Schaffer, 1988). However, if this is the case phosphorylation would not seem to be essential for Vmw175 transcriptional regulation as measured in these assays. This conserved region of the protein may be important for other functions in the infected cell, or perhaps the function of this region is partially duplicated by other regions of the protein.

Proteins expressed from plasmids deleted towards the carboxyl terminus of the reading frame, pD11 and pD12, result in severely decreased transactivation although insertions round this region do not. Deletion mutant pD9, which removes much of homology region C, is also deficient in transactivation, which is consistent with the phenotype of insert pI29 at amino acid 934. However, all three of these deletion mutants (pD9, pD11 and pD12) can repress the IE3 promoter. It is not obvious why insertion at 934 should reduce the ability of the protein to repress whereas deletion of this region does not.

Unexpectedly, several larger deletions such as pD4 which spans region 4, have greater transactivation activities than the smaller deletions in this region. It is even more difficult to understand why the out of frame deletions, pD2 and pD7, encode more competent transactivators than their daughters which have been put back in frame (and regain the epitope for 58S). These data suggest that the relationship between the structure and function of the C-terminal third of Vmw175 is particularly complicated.

All of the cytoplasmically localized mutants seem to be impaired in their repression activity, although three of them are moderate transactivators (pD4, pD6 and pD8); possibly they leak into the nucleus at levels sufficient to transactivate but not sufficient to repress. If this truly

indicates that a different concentration of Vmw175 is required for expression of each phenotype, then this would imply that each activity functions through a different mechanism. The out of frame deletions pD2 and pD7 have lost the putative nuclear localization signal but also the epitope for monoclonal 58S, so it was not possible to determine their cellular location. The enigma that they are better transactivators than their in-frame derivatives, pD2F and pD7F, may be explained if they enter the nucleus more efficiently, perhaps due to fortuitous localization signals in the out of frame portion. Alternatively, and perhaps more intriguingly, the downstream domains regained by the in-frame daughters may be acting to inhibit transactivation in these abnormal proteins.



SECTION 3D: DETERMINATION OF THE SITE-SPECIFIC DNA BINDING ACTIVITY OF THE PLASMID-BORNE MUTANTS OF IE GENE 3.

Whilst most of the HSV-1 IE proteins bind to DNA to some extent *in vitro* (Hay and Hay, 1980) Vmw175 is the only one known to interact with specific target sequences which include a proposed consensus binding sequence ATCGTC (Beard *et al.*, 1986; Faber and Wilcox, 1986). Vmw175 has been shown to bind to this sequence in the promoter regions of the IE1, IE3 and glycoprotein gD genes (Faber and Wilcox, 1986a; Kristie and Roizman, 1986a,b; Muller, 1987) but has also been reported to bind to several viral DNA fragments which lack the consensus (Kristie and Roizman, 1986a; Michael *et al.*, 1988). Evidence from a variety of laboratories suggests a correlation between the ability of Vmw175 to bind ATCGTC at the transcriptional start site of IE gene 3, and autoregulation of this promoter.

This section describes the use of nuclear extracts, made from cells transfected with the insertion and deletion mutant plasmids described in Section 3A, in gel retardation DNA binding assays in order to define the regions of Vmw175 involved in binding to the specific Vmw175 DNA binding site present at the transcriptional start (cap) site of IE gene 3. The results show that the regions of Vmw175 required for DNA binding closely correspond to those involved in transcriptional regulation. In particular, a region crucial for site specific DNA binding lies within amino acids 275-495.

1. Characterization of the site-specific DNA binding activity of Vmw175.

1.1 Vmw175 in nuclear extracts of virally infected HeLa cells forms a complex with the ATCGTC consensus binding-site located at the IE gene 3 cap site.

As described by Muller (1987) an end-labelled 45bp AvaI/BamHI DNA fragment spanning the IE3 cap site can be used to assay Vmw175 site-specific DNA binding activity using the gel retardation assay (Garner and Revzin, 1981). Cells were infected at a moi of 5pfu per cell and nuclear extracts prepared 5h after a 1h absorption period by a

procedure modified from that of Dignam et al., (1983) (Materials and Methods, Section 3B13). Nuclear extracts were incubated for 20min with the DNA probe and the protein-DNA complexes were then resolved on 4% polyacrylamide gels. Non-specific DNA binding activity was eliminated by the addition of lug polydI:polydC to the binding reactions.

Proteins present in mock infected cell extracts bind to the IE3 probe to give two specific complexes "b" and "c" (Figure 19A, tracks 1 and 2). A single major, viral specific, retarded complex "a" is detected after incubation of the probe with infected cell nuclear extract for 20 minutes at 20^{o}C and resolution by gel retardation (Figure 19A, tracks 3 and 4). This complex is further retarded "a^{+}" after additional incubation with the Vmw175 specific monoclonal antibody 58S (Showalter et al., 1981) which recognizes an epitope near to the C-terminus of Vmw175 (Paterson and Everett, 1988a; Section 3B). These complexes were not formed using extracts made from mock infected cells nor from cells infected with an HSV-1 mutant which produces no Vmw175, in1411 (Russell et al., 1987b) (Figure 19A). The complex was also formed by extracts made from cells infected with dl1403, a mutant lacking both copies of IE gene 1 which encodes Vmw110 (Stow and Stow, 1986).

Recognition of the protein-DNA complex "a" by two Vmw175 directed rabbit antisera in addition to the monoclonal antibody 58S provides further evidence that this complex contains Vmw175 (Figure 20). The antiserum 19825 is raised against a synthetic nonapeptide corresponding to the carboxyl terminus of Vmw175 (kindly provided by Dr M.Frame) whilst the antiserum 2180 V was raised against bacterially expressed fusions of fragments of Vmw175 to beta-galactosidase (kindly supplied by Dr A.Buchan, University of Birmingham). No other HSV proteins could be detected in the complex using monoclonal antibodies (against the major DNA binding protein) or antipeptide antisera (against Vmw110 and Vmw63, Figure 20). It therefore seems unlikely that the complex contains other viral gene products and indeed Vmw175 is the only viral gene product necessary for production of the complex. Low amounts of the antiserum

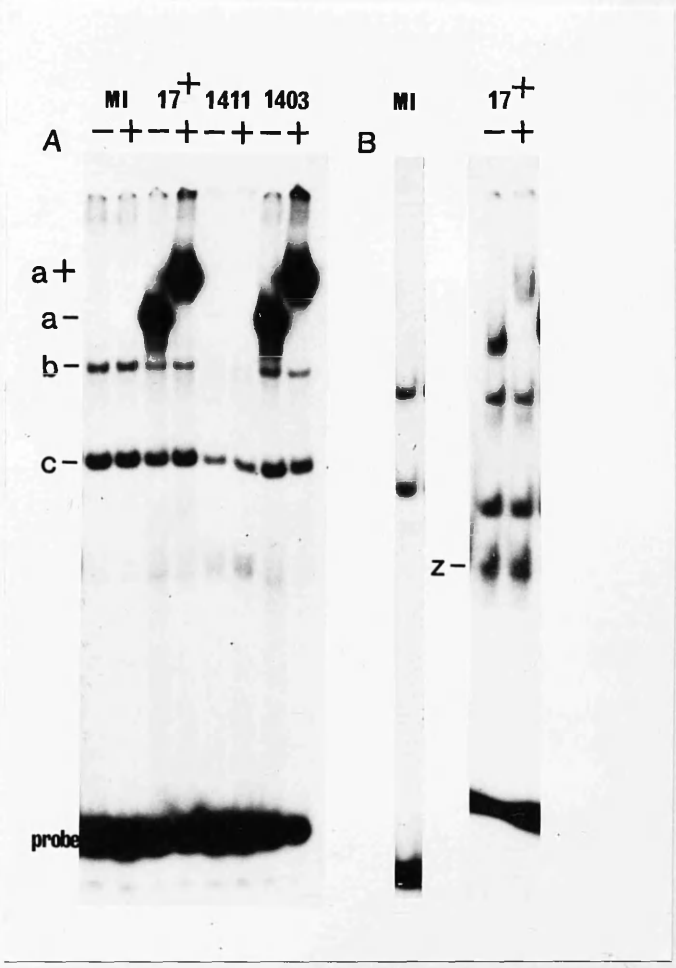


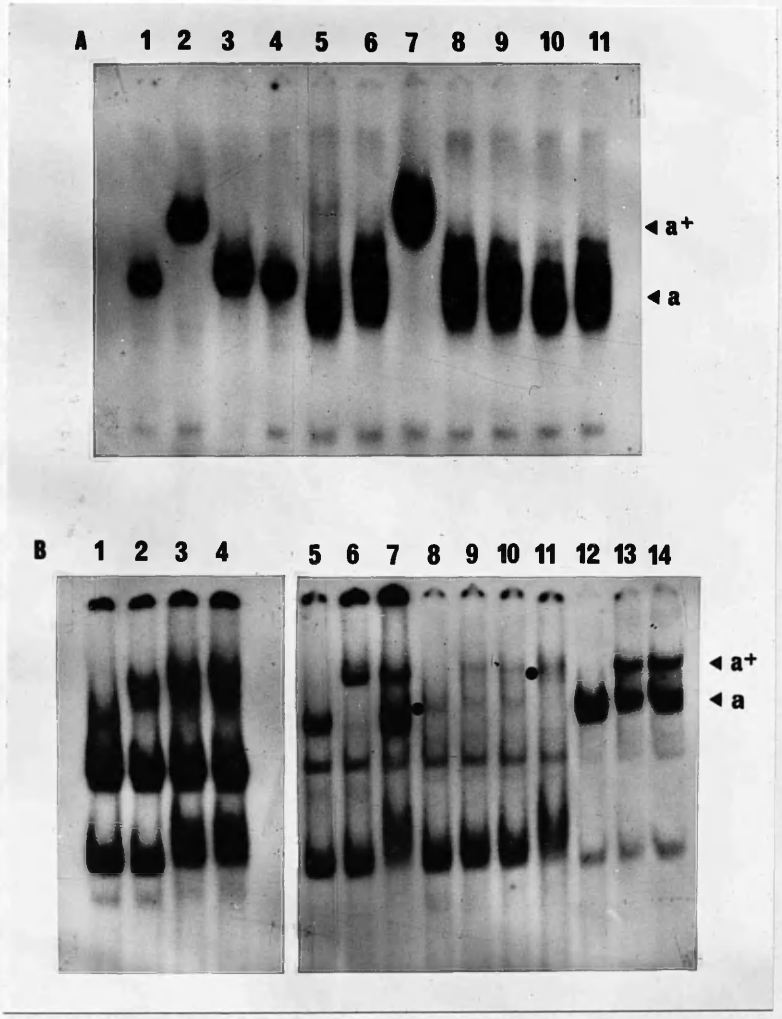
Figure 19: Vmw175 binds to the IE gene 3 transcriptional start site. Autoradiograph of a gel retardation experiment showing the protein-DNA complexes formed using nuclear extracts of HSV-1 infected cells bound to a DNA probe spanning bases -17 to +27 of the HSV-1 IE gene 3. Assays were performed as described in the Materials and Methods, with (+) or without (-) addition of monoclonal antibody 58S. Assays perfomed at (A) 20°C or (B) 40°C. MI= mock infected, 17^+= HSV-1 strain 17^+, 1403= HSV-1 mutant dl1403, 1411= HSV-1 mutant in1411, a= retarded complex containing Vmw175, a^+= complex a further retarded with antibody, b and c are complexes containing host factors, z= novel retarded band, probe= unbound DNA probe.

Figure 20: Monoclonal and polyclonal antibodies against Vmw175 recognize and specifically retard the Vmw175/DNA complex. (A) and (B) DNA binding assays were performed as described in Materials and Methods, using 1ul of nuclear extract of HSV-1 infected cells bound to the 44bp IE3 cap site probe at $0^{o}C$ for 20min, and then further incubated with antisera for 10min at $0^{o}C$. A1-11, B1-4 and B12-14 extracts of cells infected with wt 17^{+}; B5-7 infected with I15HBC (see Section 3E3); B8-11 cells transfected with p175.

Antisera used:

A1	none	B1	none
A2	1ul (1/20) 58S anti-Vmw175 McAb	B2	1ul (1/50) 58S
		B3	2ul 2180V anti-Vmw175
A3	1ul anti-ICP8 McAb	B4	4ul 2180V
A4	1ul anti-gD McAb	B5	none
A5	3ul 11666 anti-Vmw175 (N_t)	B6	1ul (1/50) 58S
A6	3ul 18817 anti-Vmw175 (C_t)	B7	2ul 2180V
A7	3ul 19825 anti-Vmw175 (C_t)	B8	none
A8	3ul 18815 anti-Vmw65 (C_t)	B9	0.5ul 2180V
A9	3ul 14711 anti-Vmw110 (C_t)	B10	1ul 2180V
A10	3ul 13963 anti-US9 (C_t)	B11	2ul 2180V
A11	3ul 14996 anti-RR_2 (C_t)	B12	none
		B13	0.5ul 2180V
		B14	1ul 2180V

Only the top portion of the autoradiograph is shown. The results show that only antisera directed against Vmw175 recognize the Vmw175/DNA ("a") complex and further retard the mobility of the complex (to "a^{+}"). Only one preparation of anti-(carboxy-terminal)-peptide antiserum bound to the complex, the (older) amino-terminal directed antipeptide serum did not recognize the complex. Incubation with the rabbit antisera (except 2180 V) results in a broader and slightly faster migrating complex band. Faint bands are highlighted in tracks 8 and 11.


A
1 2 3 4 5 6 7 8 9 10 11
a+
a
B
1 2 3 4
5 6 7 8 9 10 11 12 13 14
a+
a

2180 V shifted only part of the complex "a".

An apparently viral specific complex, labelled "z", of much greater mobility was occasionally formed (Figure 19B), especially if the binding reaction was carried out at elevated temperatures. This complex was not further retarded by the antibody 58S, indicating that it might be formed by a protein unrelated to Vmw175, or, by a proteolytic fragment of Vmw175 lacking the C-terminal 58S epitope. (A complex resembling "z" was also seen when binding was assayed in the presence of high salt concentration at elevated temperatures, see Figure 44B).

1.2 Vmw175 in nuclear extracts made from transfected cells forms an authentic complex with the IE gene 3 cap site.

Nuclear extracts prepared from cells transfected with the plasmid p175, which express Vmw175, form a specific complex with the IE3 cap site when assayed by gel retardation (Figure 21A). The complex is essentially identical to that formed using extracts of virally infected cells and is recognized by the anti-Vmw175 antibody 58S. However, transfected cell extracts occasionally formed two specific complexes which were both recognized by 58S (Figure 21B). The smaller complex (a') could be formed by an abnormally processed form of Vmw175 or it might lack an unknown factor. It usually appeared when the overall efficiency of the transfections was poor and could reflect a lower level of Vmw175 in the transfected cells.

That cells transfected with p175 formed a complex identical to that formed with infected cell extracts indicates that Vmw175 is the only viral protein present in the complex. The presence of other HSV-1 genes in the transfection had no effect on the nature of complex formed, but addition of the gene encoding Vmw63 did increase its amount (Figures 21A,B). However, Vmw63 itself did not form a complex with this DNA probe, and the addition of an extract containing Vmw63 to one containing Vmw175 did not enhance binding (Figure 21C). Rather the effect of Vmw63 seemed to be on expression of Vmw175, as the extract made from cells transfected with p175 and p63 together had 2.5 fold more Vmw175 than the extract from cells transfected

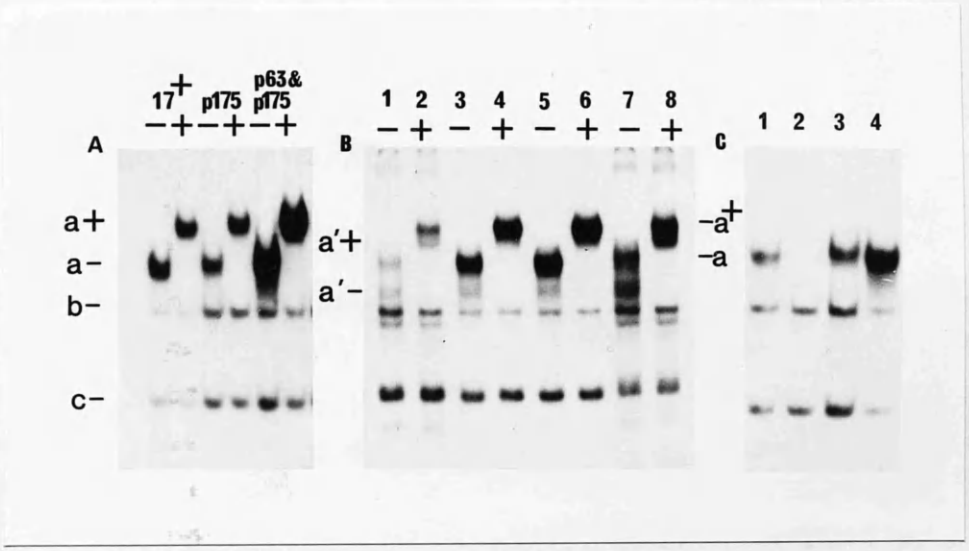


Figure 21: Nuclear extracts containing Vmw175 expressed in cells transfected with plasmid p175 form an apparently identical DNA complex to that formed by Vmw175 present in infected cell extracts. (A) Binding assays were carried out as described in Figure 19 using extracts of cells infected or transfected as shown. Unbound probe has been cut off the bottom portion of the gel. Plasmids used express Vmw175 (p175), Vmw110 (p111), Vmw63 (p63) and ICP8 (pGX38). (B) Cells were transfected with p175 and the following plasmids: lanes 1 & 2, p111; 3 & 4, p111 and p63; 5 & 6, p111, p63 and pGX38; 7 & 8, p175 alone. a'= secondary complex, shifted to a'+ with antibody. (C) Addition of extracts containing Vmw63 does not enhance Vmw175 DNA binding. Lane 1, p175; lane 2, p63; lane 3 mix of extracts used in 1 & 2, lane 4, cotransfection of p175 and p63.

with p175 alone, as determined by ELISA.

1.3 Vmw175 also forms a complex with the ATCGTC consensus binding-site located in the gD promoter.

Vmw175 has been reported to bind to a copy of the ATCGTC consensus binding site present at bases -101 to -106 in the HSV-1 gD promoter (Faber and Wilcox, 1986a). In order to investigate this activity in our assay system a XhoI-HindIII probe fragment was prepared from the plasmid pRED112 (Everett 1983) which spans bases -109-+11 of the gD promoter. Nuclear extracts prepared from virally infected cells bound to form a novel complex with this probe when assayed by gel retardation (Figure 22a). This complex was larger than the IE3 complex, probably indicating that proteins other than Vmw175 were also bound to the fragment; the ability of the antibody 58S to recognize and retard this complex confirmed the presence of Vmw175 in the complex. When a short, 26bp, synthetic probe spanning the gD promoter binding site (bases -111 to -90, with single-stranded 5' TCGA extensions) was used in this assay, a complex of similar size to that formed with the 44bp IE3 probe was formed by nuclear extracts of either virally infected or p175 transfected cells (Figure 22b). This demonstrates that Vmw175 is the only viral protein present in this complex, and indeed probably only one protein complex could form on a probe this size.

1.4 The DNA binding region of Vmw175 forms a protease resistant domain.

In order to investigate whether the complex "z", described in Section 3D1.1 above, was related to the Vmw175 complex and perhaps formed by a proteolytic breakdown product of Vmw175 binding to DNA, proteolysis experiments were performed. Commercial proteinase K enzyme was incubated with binding reactions for 10 minutes at room temperature, before resolving the products on a standard retardation gel. Concentrations of proteinase K between 1 and 10ng per reaction were found to degrade the specific Vmw175-DNA complex "a" to a much smaller complex "p" (Figure 23A). The same complex "p" is formed whether protease

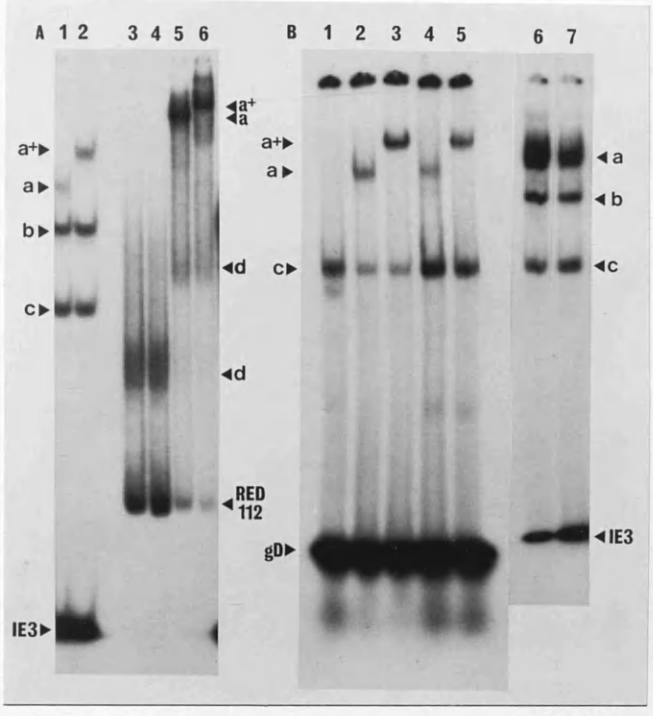


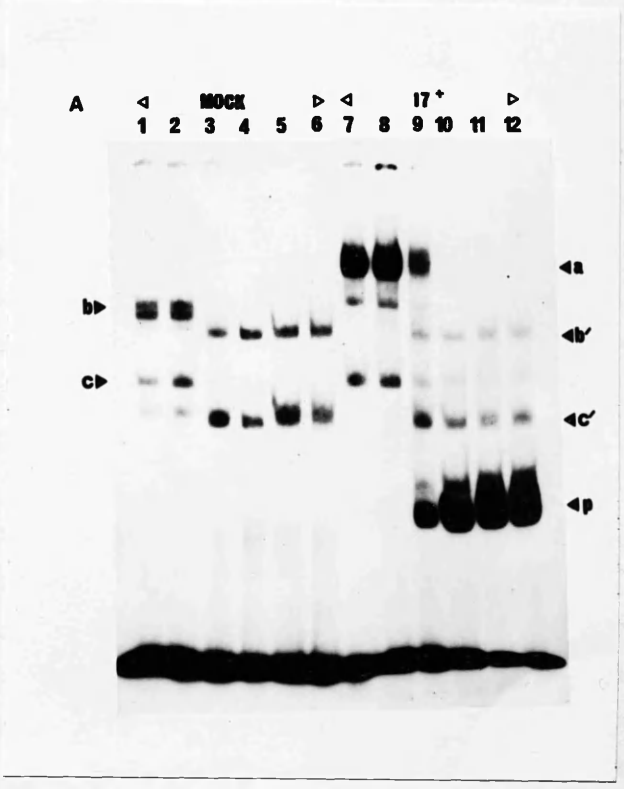
Figure 22: Vmw175 binds to an ATCGTC element upstream of the gD promoter to form a complex similar to that formed with IE3 cap site. (A) An end-labelled probe spanning the gD promoter was prepared by filling in the XhoI-HindIII fragment (-109-+11) from the plasmid pREDl12. This was incubated (at 20°C) with mock (lanes 3 & 4) or HSV-1 strain 17^+ infected nuclear extracts (lanes 5 & 6) and compared by gel retardation to the Vmw175/IE3 cap site complex (lanes 1 & 2). Reactions 2, 4 & 6 are further incubated with the antibody 58S. As can be seen in lanes 5&6, infected extracts form a virus specific complex ("a") with the gD promoter, which contains Vmw175. However, other proteins are probably bound to this DNA probe as the complexes "a" and "a^+" are larger than those formed on the IE3 probe. ("d": non-Vmw175 containing complexes; "REDl12": unbound probe). (B) A 26bp double stranded oligonucleotide spanning residues -111 to -90 of the gD promoter including the ATCGTC consensus sequence, with 5' TCGA single-stranded extensions, was end-labelled and assayed for Vmw175 binding at 20°C using mock infected (lane 1), pl75-transfected (lanes 2&3) or HSV-1 17^+ infected extracts (lanes 5&6). The complexes formed were compared by gel retardation to the Vmw175/IE3 cap site ("a") complex (lanes 5&7). Reactions 3 and 5 were further incubated with antibody 58S. The results show that Vmw175 forms a similar specific complex with the short gD promoter oligonucleotide to that formed with the short IE3 cap site probe. The gD probe is also bound by a cellular factor to form complex "c", but not complex "b".

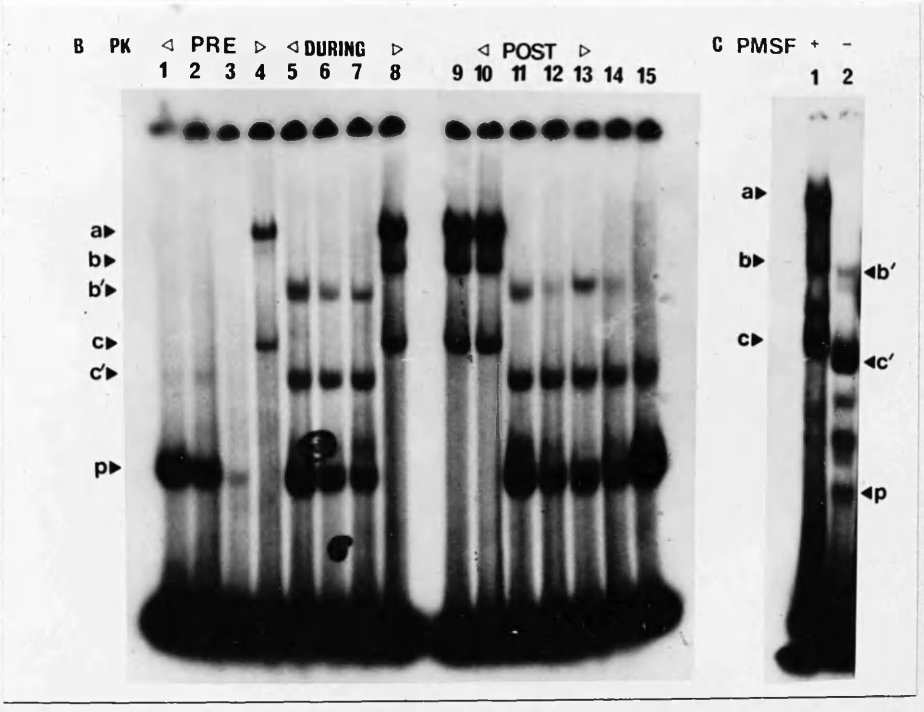
Figure 23: A protease resistant domain of Vmw175 binds to DNA.

(A) Mock or 17^+ infected extracts were assayed for IE3 cap site DNA binding as previously described, but further reacted with commercial protease K (PK) at $20^{o}C$ for 10min before electrophoresis. The amounts of PK used were: lanes 1&7: 0ng; lanes 2&8: 1ng; lanes 3&9: 10ng; lanes 4-6&10-12: 20ng. A novel viral specific complex "p" was formed, presumably by proteolytic degradation of complex "a". In addition the host-factor specified complexes b and c were degraded to give the faster migrating complexes b' and c'. The protease inhibitor PMSF was added to reactions 5, 6, 11 & 12 before further incubation with pre-immune rabbit antiserum (5 & 11) or with the anti-Vmw175 antiserum 2180V (6 & 12). Complex "p" did not appear to be recognized by this antiserum.

(B) Reactions were performed as in (A) using 17^+ infected extracts but in the case of reactions 1-4 the PK digestion was performed prior to addition of the DNA probe (and PMSF) and in the case of reactions 5-6 DNA binding was performed in the presence of PK. Amounts of PK used: lane (9): 0ng; lanes (4,8,10): 1ng; (3,7,11): 10ng; (2,6,12): 20ng; (1,5,13-15): 100ng. Reactions 14 and 15 were further incubated with the anti-Vmw175 antisera 58S (14) and 19825 (15) in the presence of PMSF. The results show that a protease resistant domain of Vmw175 can bind to DNA, and that this domain is protease resistant in the presence or absence of DNA.

(C) Identical DNA binding reactions of 17^+ infected extracts to the IE3 probe were performed in 1 & 2 followed by PK digestion prior to electrophoresis, however, in the case of reaction 1, PK digestion was performed in the presence of PMSF, which is shown to inhibit PK activity.


A
MOCK
17+
1 2 3 4 5 6 7 8 9 10 11 12
a
b
b′
c
c′
p


B PK
PRE
DURING
POST
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
a
b
b′
c
c′
p
C PMSF + −
1 2
a
b
b′
c
c′
p

digestion is performed prior, during or after the DNA binding reaction (Figure 23B) indicating that the DNA binding region of Vmw175 forms a distinct structural domain. Complexes "b" and "c", which are formed by cellular DNA binding factors, are degraded to lesser extent to give new bands "b'" and "c'" when either virally or mock infected extracts are assayed (Figures 23A,B). Complex "p" is not formed by mock infected nuclear extracts (Figure 23A). It seems likely that complex "z" which occassionally appears in the absence of proteinase K treatment (see Figure 19B) may be a proteolytic product of band "c" and unrelated to Vmw175.

Proteinase K activity could be inhibited by the addition of 0.2mM PMSF to the incubations (Figure 23C) which allowed antibody shift experiments to be carried out on complex "p" (Figure 24). The proteolytic fragment of Vmw175 present in complex "p" did not appear to be recognized by any of the antisera. The 58S and 19825 antisera are directed against carboxyl terminus epitopes of Vmw175, which are presumably free to be cleaved away from the protein-DNA complex; only serum 2180 V might be directed against epitopes in the DNA binding domain, but it too failed to recognize complex "p" (Figure 24).

1.5 The Vmw175-DNA complex is highly salt-stable.

As a consequence of the strong ionic interactions between a site-specific DNA binding protein and its DNA target such complexes are generally stable at relatively high salt concentrations. DeLuca and Schaffer (1988), however, have reported that Vmw175-DNA complexes assayed in their studies are highly salt-labile. In contrast to their observations, the Vmw175-IE3 CAP site complex investigated in these studies proved to be highly salt stable and was still detectable when assayed at 0.5M NaCl (Figure 25A,B). Complex "c" was also reasonably salt stable, but when the binding reaction was performed at 0.2M NaCl complex "b" was not detectable. When the binding reactions were carried out at 39^{o}C, complex "a" was only marginally more salt labile (Figure 25B).

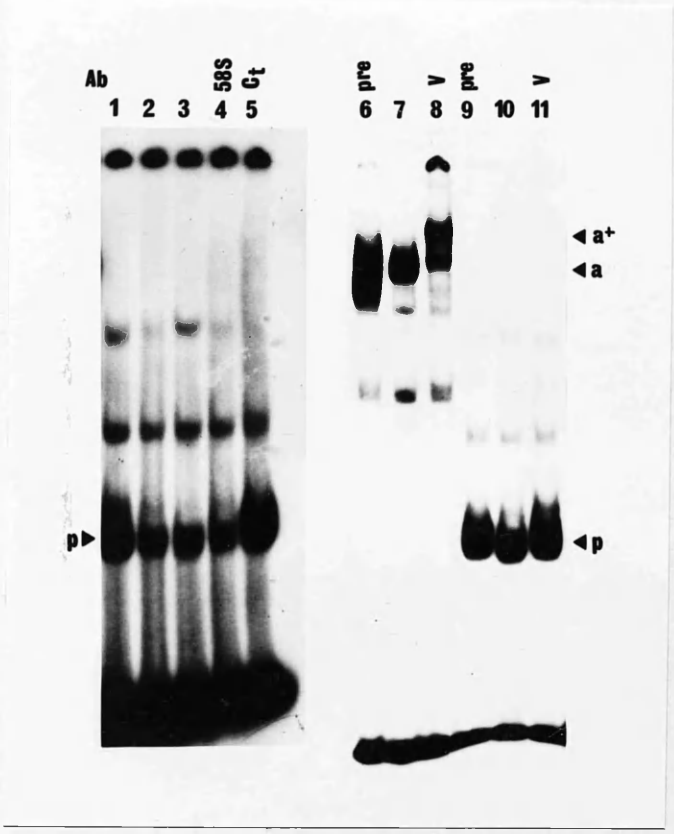


Figure 24: The proteolytic fragment of Vmw175 which binds to DNA is not recognized by available antisera. Reactions were performed as described as for Figure 23. Lanes 1-5 are as Figure 23B 11-15, lanes 6-8 no PK, lanes 9-12 100ng PK following DNA binding. Lanes 6 & 9 were bound to pre-immune rabbit antiserum, lanes 8 & 11 to 3ul antiserum 2180V. The results show that complex "p" is not recognized by any of the three available anti-Vmw175 antisera.

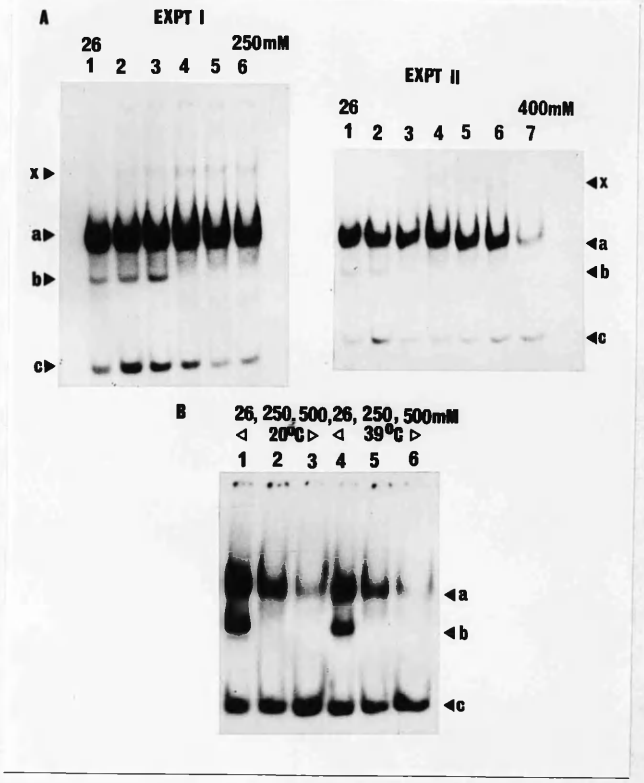


Figure 25: The Vmw175/DNA complex is highly salt-stable. (A) The results of two separate DNA binding experiments using 17^{+} infected extracts incubated with the IE3 cap site probe in the presence of increasing concentrations of NaCl are shown. mM NaCl in experiment I: (1) 26 (2) 76 (3) 100 (4) 150 (5) 200 (6) 250; in experiment II: (1) 26 (2) 100 (3) 200 (4) 250 (5) 300 (6) 350 (7) 400. The results show that the Vmw175 complex "a" and the host-cell factor complex "c" are highly salt stable. A novel uncharacterized complex "x" appears at high salt concentrations.
(B) The salt-stability of the Vmw175-DNA complex was unaffected by performing the binding reaction at an elevated temperature of 39°C.

1.6 Vmw175 site-specific DNA binding activity in different cell-types.

It was of interest to investigate the DNA binding activity of Vmw175 in extracts of different cell-types in order to correlate it with the transcriptional regulation experiments, which were performed in WS HeLa and Vero cells, and because the activity of Vmw110 and 175 has been shown to vary in different cell-types (Everett, 1988b). Flow HeLa cells were routinely used to prepare extracts for DNA binding experiments as the extracts of transfected Flow HeLas were apparently more active than those of WS Helas, even though ELISA experiments indicated that they contained similar amounts of Vmw175 (Figure 26B). This suggests that complex formation is not directly related to the amount of ELISA-detectable Vmw175 in an extract. This could reflect differences in the stability or modification of Vmw175 in different cell-types, or perhaps differences in other cell factors involved in complex formation. However, infected cell extracts from Flow and WS HeLas seemed to behave identically (Figure 26A). Vmw175/IE3 cap site binding activity was not detectable in either transfected or infected nuclear extracts of Vero cells (Figure 26A,B) although Vmw175 was detected in the infected cell extracts by ELISA and Vmw175 has been shown to repress expression of pIE3CAT in these cells (Section 3C2). The failure to detect DNA binding *in vitro* may not necessarily reflect a failure to bind *in vivo* as the protein may not be stable upon extraction from nuclei.

2. The regions of Vmw175 important for DNA binding closely correspond to those important for transcriptional regulation.

2.1. Analysis of insertion mutants.

The DNA binding activity of the insertion mutants described in Section 3A was investigated in gel retardation assays using nuclear extracts made from Flow HeLa cells transfected with each of these 39 plasmids.

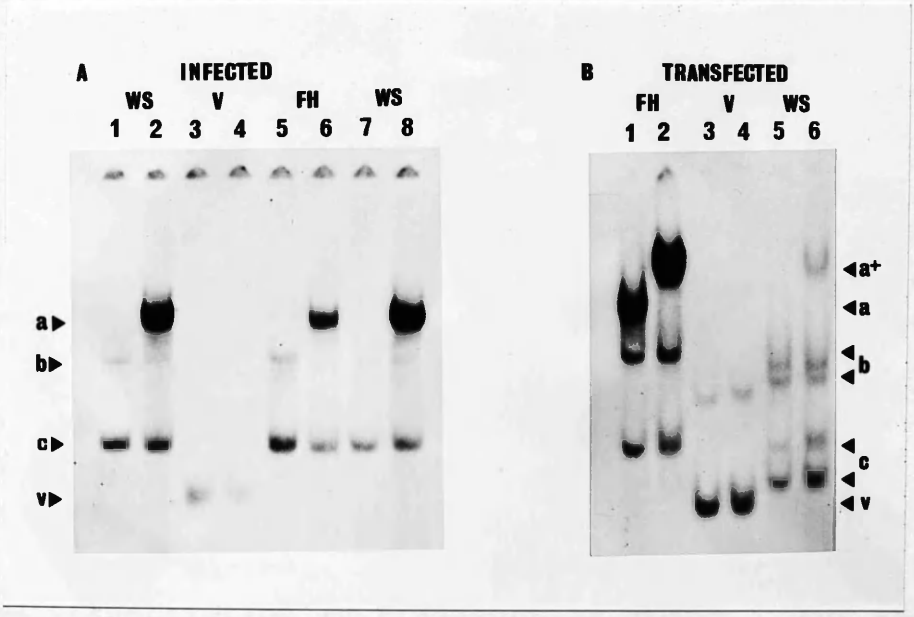
Nuclear extracts were prepared from $7x10^6$ transfected cells and one twentieth (4ul) of each was incubated with the IE3 cap site probe in a 30ul reaction volume at 20^oC for 20

Figure 26: Similar Vmw175/DNA complexes are formed by extracts of transfected and infected HeLa cells, but are not detected with extracts of Vero cells. Extracts of three infected (A) and transfected (B) cell-types were assayed for Vmw175/IE3 cap site binding as detailed below. The amount of ELISA detectable Vmw175 in each extract was also determined.

lane	cells	extract	ELISA (relative amount Vmw175)	Antibody shift (58S)
A1	WSHeLa	MI	0.0	-
A2	WSHeLa	17^+	0.31	-
A3	Vero	MI	0.0	-
A4	Vero	17^+	0.48	-
A5	FHeLa	MI	0.0	-
A6	FHeLa	17^+	1.88	-
A7	WSHeLa	MI	n.d.	-
A8	WSHeLa	17^+	n.d.	-
B1	FHeLa	p175	0.88	-
B2	FHeLa	p175	0.88	+
B3	Vero	p175	0.05	-
B4	Vero	p175	0.05	+
B5	WSHeLa	p175	0.70	-
B6	WSHeLa	p175	0.70	+

(FHeLa: Flow Hela; MI: mock infected extracts; 17^+: HSV-1 strain 17^+ infected extract; p175: p175 transfected extract; v: novel non-viral-specific complex formed by Vero cell extracts.)

The results show that identical complexes are formed by transfected and infected extracts of both types of HeLa cells, but that complex formation by Vmw175 in WSHeLa transfected cells was much less efficient. No Vmw175/DNA complex could be detected using extracts prepared from Vero cells, although infected Vero cell extracts contained detectable levels of Vmw175.


A
INFECTED
WS
V
FH
WS
1 2 3 4 5 6 7 8
a
b
c
v
B
TRANSFECTED
FH
V
WS
1 2 3 4 5 6
a+
a
b
c
v

minutes, then further incubated with 1ul 1/100 dilution of monoclonal 58S prior to separation on 4% polyacrylamide gels. The resulting autoradiographs are shown in Figure 27. The antibody shift was carried out in order to clearly resolve the Vmw175 complex from the host factor complexes "b" and "c", although in some cases the addition of antibody seemed to stabilize the degree of complex formation (results not shown). In addition to the major "a^+" complex, the minor "z" complex was also occasionally present, but is probably not related to Vmw175 (Section 3D1.4). The binding reactions were carried out at 20^{o}C; at higher temperatures the binding of wild type Vmw175 was significantly reduced, although temperature sensitive mutants can be studied in this manner (see Section 3F6).

The amount of Vmw175 in each extract was measured by ELISA and compared to the p175 control transfection to ensure that the transfections were working with comparable efficiency and that the Vmw175 polypeptide was present in the extract. These results, together with a summary of the binding results in Figure 27, are presented in Table 9, which also summarizes the map positions and transcriptional regulation results (Sections 3C1 and 3C2).

Table 9 shows that mutants impaired in their ability to bind to the IE3 cap site <u>in vitro</u> broadly correspond to those impaired in their ability to transactivate and to repress transcription in transfection assays. For example insertion mutants pI12 to pI18 (with the exception of pI13) are all significantly reduced in the transactivation and repression assays and also in their ability to bind to the IE3 cap site. This is especially true of mutants pI14-pI18. Indeed it is clear that this portion of the protein, (region 2; amino acids 315-484) is of critical importance for both transcriptional control and DNA binding. Only one mutant in region 2, pI13, can efficiently bind to this DNA probe, but fails to express either repression or transactivation activity.

Insertion mutants throughout the majority of region 1 are virtually unimpaired in DNA binding activity or transcriptional regulation. However, two mutants, pI10 and pI12, situated at the end of region 1 close to the junction

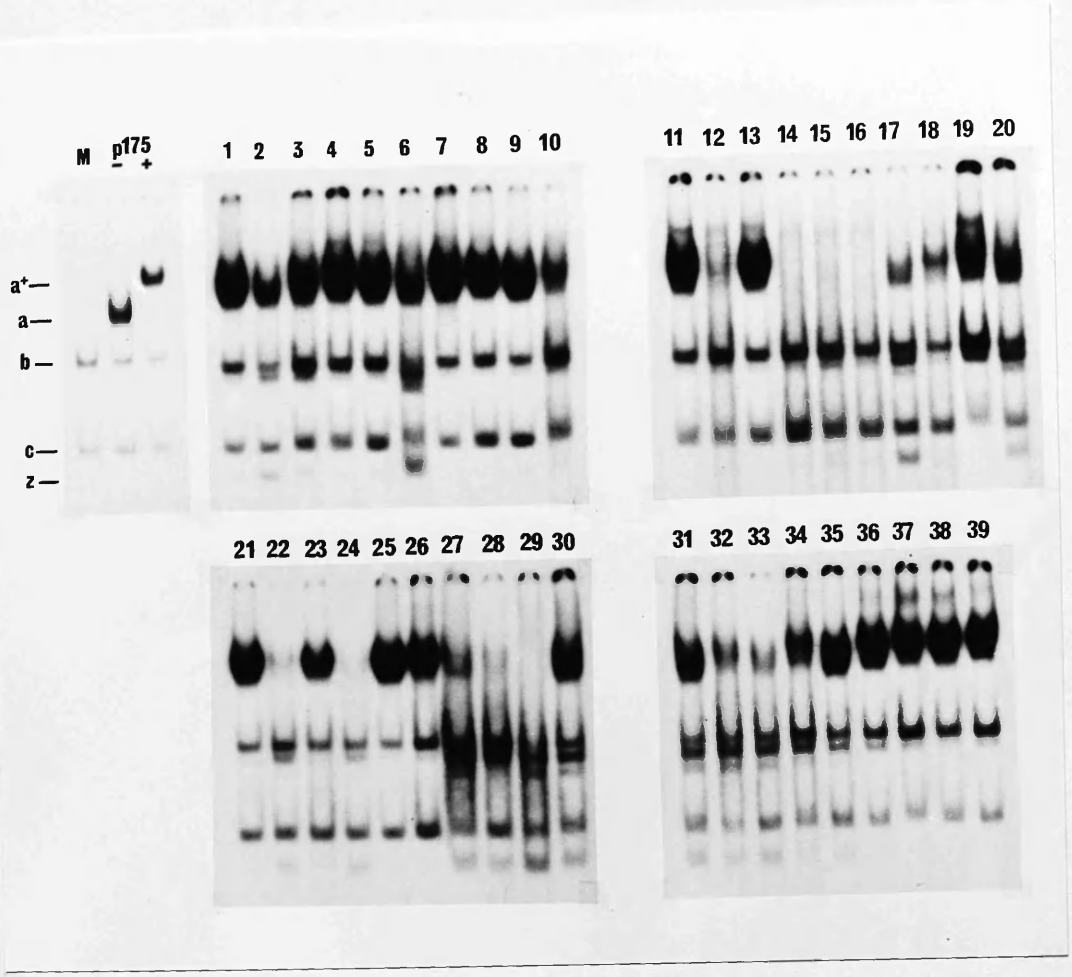


Figure 27: DNA binding assays using extracts of cells transfected independently with each of the 39 insertion mutants pI1-pI39. All assays were incubated with antibody 58S except M (mock transfected) and p175/- (positive control). Complexes a, a^+, b, c and z are as described in Figure 19. The results are summarized in Table 9.

Legend to Table 9.

a The site (amino acid) of linker insertion.

b The activation of the gD promoter (using pgDCAT in conjunction with Vmw110 provided by p111) is given as a percentage of that obtained in parallel experiments with p175. The mean activity (x) and the standard error of the mean (SEM) are shown, reproduced from Table 7.

c The qualitative ability to repress the IE3 promoter of pIE3CAT compared to p175 in cotransfection titration experiments, reproduced from Table 7. Repression activity is scored from - (essentially no activity) to +++ (wild type activity).

d The ability of nuclear extracts made from HeLa cells transfected with each of the mutants to bind to the IE3 cap site. Binding activity is scored from visual inspection of Figure 27: - no binding, +/- barely detectable, + to +++ increasing binding activity.

e Relative amount of Vmw175 in each nuclear extract determined by ELISA. (p175=100)

f Further experiments with this mutant did produce detectable amounts of pI29 Vmw175 as described in the text and Figure 28.

Table 9: A summary of the positions and activities of the insertion mutants.

Mutant	Insert Site[a]	Transact. activity[b] x (SEM)	Repress. activity[c]	DNA binding activity[d]	Relative amount[e] of Vmw175
pI1	12	35.1 (11.7)	+++	+++	288
pI2	71	53.4 (13)	++	++	203
pI3	83	49.6 (6.7)	++	+++	88
pI4	123	65.6 (12.8)	++	+++	241
pI5	130	43.8 (6.5)	+++	+++	106
pI6	137	54.4 (11.8)	++	+++	103
pI7	161	44.5 (10.5)	+++	+++	297
pI8	229	45.7 (8)	+++	+++	127
pI9	252	127 (43.2)	+++	+++	263
pI10	275	25.8 (4.6)	-	++	142
pI11	292	30.8 (6.3)	++	+++	276
pI12	310	20.0 (4.4)	+	+/-	97
pI13	324	10.0 (2.9)	-	+++	226
pI14	329	4.9 (0.9)	-	-	38
pI15	337	4.5 (0.9)	+	-	18
pI16	373	16.5 (2.8)	-	-	18
pI17	398	11.8 (1.9)	-	+	39
pI18	438	15.5 (4.4)	-	+	65
pI19	494	49.2 (10.4)	+++	+++	170
pI20	518	47.2 (10.2)	++	+++	85
pI21	561	43.4 (4.2)	+++	+++	294
pI22	591	26.2 (6.2)	++	+/-	206
pI23	649	23.9 (5.6)	++	++	297
pI24	681	21.1 (4.2)	++	+/-	91
pI25	774	38.2 (6.1)	+++	+++	291
pI26	787	80.9 (18.5)	+++	+++	70
pI27	803	45.9 (9.6)	++	+	126
pI28	843	10.8 (3.9)	+++	+/-[f]	15[f]
pI29	934	3.4 (0.5)	+	-[f]	0[f]
pI30	943	48.2 (12.3)	++	+++	58
pI31	1021	22.4 (3.7)	+++	+++	100
pI32	1066	10.3 (3.0)	+++	+	91
pI33	1098	30.0 (8.9)	++	+	15
pI34	1133	20.8 (2.3)	++	++	27
pI35	1139	48.2 (13.6)	++	+++	121
pI36	1195	86.9 (18)	+++	+++	218
pI37	1231	47.5 (10.3)	+++	+++	221
pI38	1236	63.1 (11.2)	+++	+++	245
pI39	1239	51.1 (8.6)	+++	+++	248

with region 2, are significantly reduced in their abilty to repress the IE3 promoter, and both are impaired in their ability to bind to the IE3 cap site, pI12 markedly so.

However, DNA binding does not show an absolute correlation with either transactivation or repression. For example two insertion mutants in region 3, pI22 and pI24, are moderately efficient transcriptional regulators, but bind to the IE3 promoter very poorly in vitro. Similarly several mutants in region 4 have markedly reduced DNA binding activity, and whilst some of these have deficient transactivation activity (pI28, pI29 and pI32) all except pI29 are fairly efficient repressors of IE3 expression. These individual descrepancies in the correlation between DNA binding and transcriptional activity may reflect differences between binding in vitro and in the nucleus of the transfected cell.

One mutant, pI29, which failed to bind DNA in Figure 27 failed to produce detectable Vmw175 in these initial nuclear extracts. However, previous studies had detected low numbers of cells expressing nuclear Vmw175 by immunofluorescence of cells transfected with pI29 (Section 3B). When the transfections were repeated using BBS instead of HBS, detectable amounts of Vmw175 could be recovered and shown to bind to the IE3 cap site probe when incubated at $0^{o}C$ (Figure 28). However, the complex formed was of reduced mobility both in the presence ("n+") and absence ("n") of antibody. Furthermore, a significant proportion of the bound DNA failed to enter the gel and remained in the well ("w"). This may indicate that the I29 mutation induces incorrect processing or folding of Vmw175, which causes it to migrate less rapidly through the non-denaturing polyacrylamide gel. Total cell extracts of cells transfected with pI29 had greater amounts of Vmw175 binding activity than nuclear extracts which may indicate that the polypeptide encoded by pI29 is deficient in nuclear localization.

It is also possible that the protein expressed by pI29 is unstable either in vivo or in vitro. The clustering of insertion sites which result in plasmids producing reduced amounts of Vmw175 in nuclear extracts (Table 9) may indicate

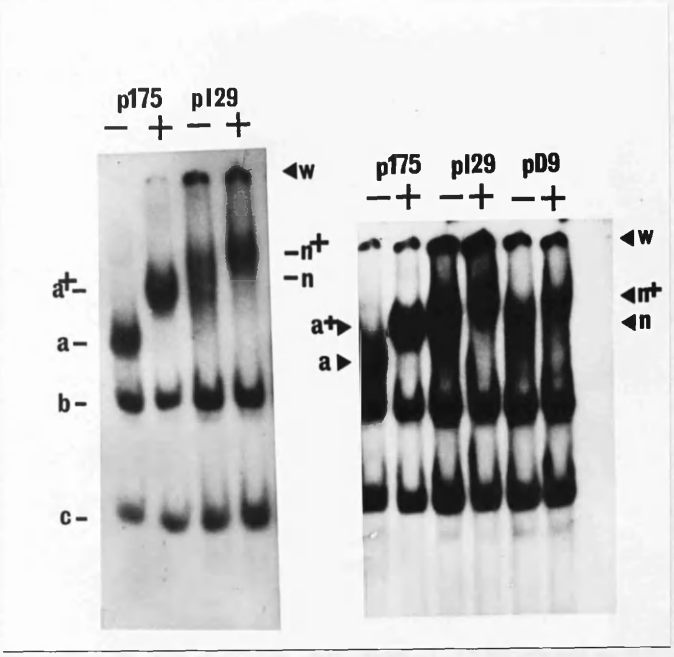


Figure 28: An abnormal complex is formed by total cell extracts of cells transfected with pI29 and pD9. Binding was performed at 0°C with (+) or without (-) addition of antibody 58S, using whole cell extracts of cells transfected with pl75, pD9 or pI29. Complexes a, a^+, b and c are as described in Figure 19, the novel complex, n_f formed by pI29 and pD9 transfected extracts is shifted to n^+ by addition of antibody. A significant amount of material (w) fails to migrate into the gel and is retained in the wells when both of the mutants are assayed in the presence or absence of antibody.

that certain regions are important for protein stablity, for example the regions of the protein defined by insertions I14-18, I28-30 and I33-34. Interestingly, mutations in the main DNA binding domain (I14-18) may result in a less stable protein. Apparently low levels of protein expression do not of themselves explain poor DNA binding since some extracts containing low levels of ELISA-detectable protein are capable of binding to DNA, for example pI34 and the deletion mutant pD9 (Table 10). It is also possible that the low ELISA measurements could be explained by failure to efficiently extract or detect mutant polypeptides if, for example, insertions effect protein solubility or presentation of the 58S epitope.

2.2. Analysis of deletion mutants.

The deletion mutants described in Section 3A were also assayed for their ability to bind to the IE3 DNA probe in gel retardation assays. Binding incubations were performed at $0^{o}C$ in order to increase the sensitivity of the assay. These results are presented in Table 10 together with the results from the transcription and immunofluorescence assays described above (Sections 3B,C) and the relative amount of ELISA detectable protein in each extract. Transfections were repeated at least three times for those extracts which failed to bind DNA and the highest ELISA measurement recorded.

DeLuca and Schaffer (1988) have previously reported that a truncated polypeptide, encoding only the first 590 amino acids of Vmw175, is able to bind to DNA under some conditions. The results presented in Table 10 and Figure 29 confirm their results and allow a more precise definition of the regions of Vmw175 unimportant for DNA binding.

Plasmids which expressed variants of Vmw175 with some DNA binding activity all have deletions which do not include amino acids 229-495, (Table 10, Figure 29). Whilst not all plasmids with deletions lying totally outwith this region express binding competent proteins (for example pD3, pD5 and pD7F) individual deletions which do not completely abolish DNA binding cover most of the rest of the gene (deletions 1, 2F, 6, 8, 9, 11, 12 and 13; Table 10). Because large

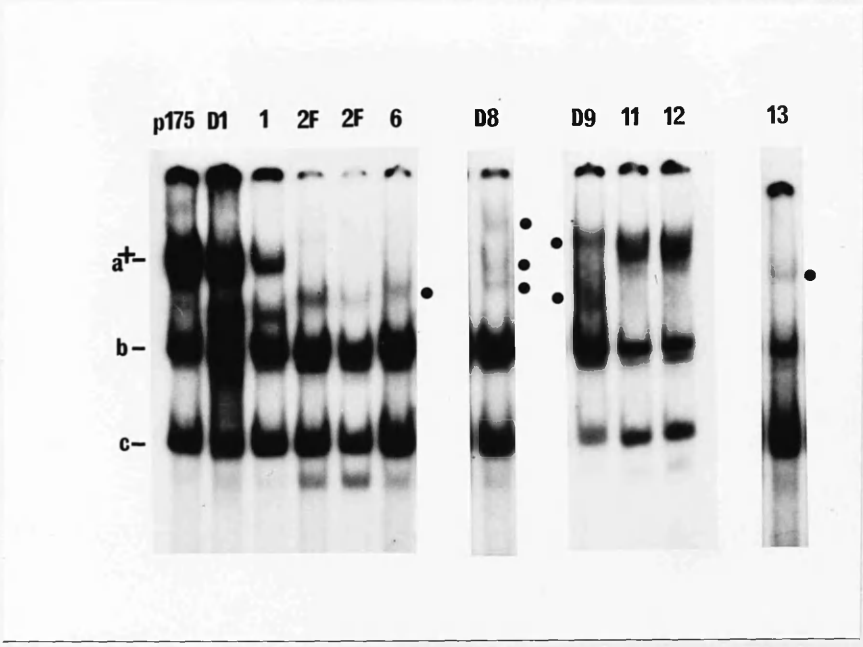


Figure 29: DNA binding by deletion mutants. Binding assays were performed at 0°C and further incubated with the monoclonal antibody 58S, using total cell extracts of cells transfected with deletion mutation plasmids pD1-15 (see Table 10). Only mutants with some detectable binding activity are shown. The wild type (p175) shifted complex (a^+) is labelled, as are the host derived complexes b and c; weak complexes are highlighted.

Table 10: A summary of the extent and activities of the deletion mutants.

Mutant	Deleted amino acids	Frame[a]	IIF[b]	Transact. act.[c] x (SE)	Repress. act.[d]	DNA binding act.[e]	Relative amount of Vmw175[f]
pD13	12-229	IN	n.d.[g]	n.d.	n.d.	weak	25
pD14	72-292	IN	n.d.	n.d.	n.d.	-	14
pD15	84-292	IN	n.d.	n.d.	n.d.	-	15
pD1	162-229	IN	nuc.	30 (4.8)	+++	+	216
pD2	495-803	OUT	-	23 (4.0)	-	-	n.d.
pD2F	496-803	IN	cyt.	5 (0.5)	+	weak	52
pD3	518-591	IN	nuc.	4 (0.5)	+++	-	107
pD4	518-1133	IN	cyt.	18 (3.3)	++	-	27
pD5	562	OUT	-	3 (1.3)	++	-	n.d.
pD6	592-774	IN	cyt.	29 (2.8)	+	weak	25
pD7	650-1066	OUT	-	27 (5.4)	-	-	n.d.
pD7F	650-1066	IN	cyt.	10 (1.5)	+	-	88
pD8	682-774	IN	cyt.	57 (5.6)	-	weak	95
pD9	934-1098	IN	nuc.	4 (0.6)	+++	+	13
pD11	1196-1239	IN	nuc.	6 (2.2)	+++	+	59
pD12	1232-1269	IN	nuc.	7 (2.2)	++	+	52

[a] Phase of reading frame 3' to deletion

[b] Cellular localization of protein as determined by immunofluorescence. nuc. nuclear, cyt. cytoplasmic, - not detectable by immunofluorecence

[c] The activation of the gD promoter as described in Table 8

[d] The qualitative ability to repress the IE3 promoter as described for Table 1

[e] The ability of whole cell extracts made from cells transfected with each of the mutants to bind to the IE3 cap site probe in gel retardation assays (at 0°C). Binding is scored simply as positive (+), negative (-) or weak.

[f] Relative amount of Vmw175 in each nuclear extract determined by ELISA. (p175=100, the results are given for the extracts used in Figure 29, or, in the case of the non-binding mutants, for extracts containing the most Vmw175; out of frame proteins could not be detected by ELISA.)

[g] n.d. not done

deletions of the protein may cause many unpredictable changes in protein stability and conformation, perhaps only those deletions which do not totally abolish DNA binding activity should be considered significant results. The results with the deletion mutants, therefore, confirm the conclusion from the insertion mutant assays that the main DNA binding domain of Vmw175 centres around region 2.

Interestingly, deletions in regions 4 and 5 (pD9, D11 and D12) give rise to somewhat larger DNA complexes than that formed by wild type Vmw175, although these deletion plasmids are predicted to express proteins of a lower molecular weight (Figures 28 and 29). As suggested above for the insertion mutant pI29 this might be caused by a partial denaturation of these mutant polypeptides *in vivo* or *in vitro*. Some proteins deficient for nuclear localization (pD2F, D6 and D8) were able to bind to the IE3 probe, albeit very weakly, when extracts were prepared from whole cells (Figure 29); nuclear extracts of these deletion mutants did not contain significant amounts of Vmw175 (results not shown). It is also noteworthy that deletions pD1 and pD13 which remove a serine rich phosphorylation site in region 1 are also able to bind DNA.

3. *DNA binding studies using an in vitro synthesized Vmw175 fusion protein.*

Recent reports that purified Vmw175 is capable of binding to DNA (for example, Michael *et al.*, 1988) raised the possibility that Vmw175 expressed *in vitro* might also be capable of binding to DNA in the gel retardation assay. In order to investigate this a plasmid expression vector was constructed which could be used to transcribe and translate the Vmw175 gene product *in vitro*. The recent success of Ace *et al.*, (1988) in expressing the HSV-1 TIF, Vmw65, *in vitro* and using this in gel retardation assays, provided a hopeful protocol to exploit. Rather than attempt to achieve efficient expression from a *de novo* construct their expression plasmid, pGEMTIF, was used to create a fusion between the first five amino acids of Vmw65 and residues 84-1029 of Vmw175, terminating in 64 residues encoded by the out of frame 3' end of the Vmw65 gene. The construction of

the plasmid expressing this fusion, pGEM175, is outlined in Section 3A4.

RNA was transcribed from EcoRI linearized pGEM175 using T7 polymerase, and a portion of this RNA translated in rabbit reticulocyte lysates, in the presence of ^{35}S-labelled methionine (Materials and Methods, Section 3B28). When the product was electrophoresed on a 9% SDS polyacrylamide gel it was found to migrate with approximately the correct mobility for a protein of 1015 amino acids, that is between Vmw175 and Vmw110 (Figure 30). However, expression of the Vmw175-Vmw65 fusion protein appeared to be much less efficient than that of Vmw65 from the parent plasmid pGEMTIF, on the basis of radioactive label incorporation.

Site-specific DNA binding to the IE3 cap-site probe was investigated using the gel retardation assay described above (Section 3D1). Labelled or unlabelled fusion protein which had been expressed *in vitro* was incubated with labelled probe in the presence or absence of mock infected cellular extracts. However, upon electrophoresis no novel retarded complexes were detected (Figure 31). The occasional detection of a complex ("y") running at a similar mobility to the Vmw175 proteolytic cleavage complex "p" (Figure 31A,B) did not seem to be dependent upon Vmw175 (Figure 31B).

The failure to detect binding activity using the *in vitro* synthesized fusion protein could be for a plethora of reasons. Firstly, on the basis of Figure 30, inadequate amounts of the protein might have been expressed with which to detect DNA binding. It may be significant that the GEM175 fusion uses the first translational initiation codon of Vmw65, whilst a second start codon at amino acid 12 may be of some importance in the translation of Vmw65, but is deleted in this fusion, (Pellet *et al.*, 1985; Dalrymple *et al.*, 1985). Secondly, the fusion constructed might have lacked regions essential for DNA binding (although the results presented in Sections 3D2 and 3D3 would not support this). Thirdly, the particular fusion we constructed might have its DNA binding activity inhibited due to abnormal folding of the protein structure, in particular the presence of a truncated carboxyl portion of the protein might be

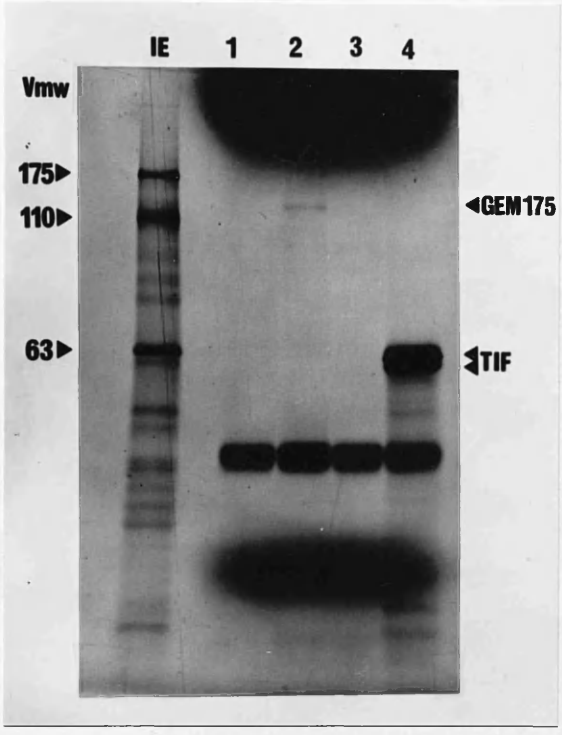


Figure 30: In vitro transcription and translation of pGEM175. 5ul of the proteins synthesized by the in vitro transcription and translation of the constructs pGEM2 (1), pGEM175 (2), pGEM571 (3), and pGEMTIF (4) were analyzed on a 9% SDS polyacrylamide gel and exposed to autoradiography. The marker lane IE is a ^{35}S-methionine labelled nuclear extract of an HSV-1 IE infection (kindly supplied by RD Everett). Whilst readily detectable amounts of Vmw65 (TIF) are produced from the pGEMTIF construct, synthesis of a protein corresponding to the GEM175 fusion is barely detectable.

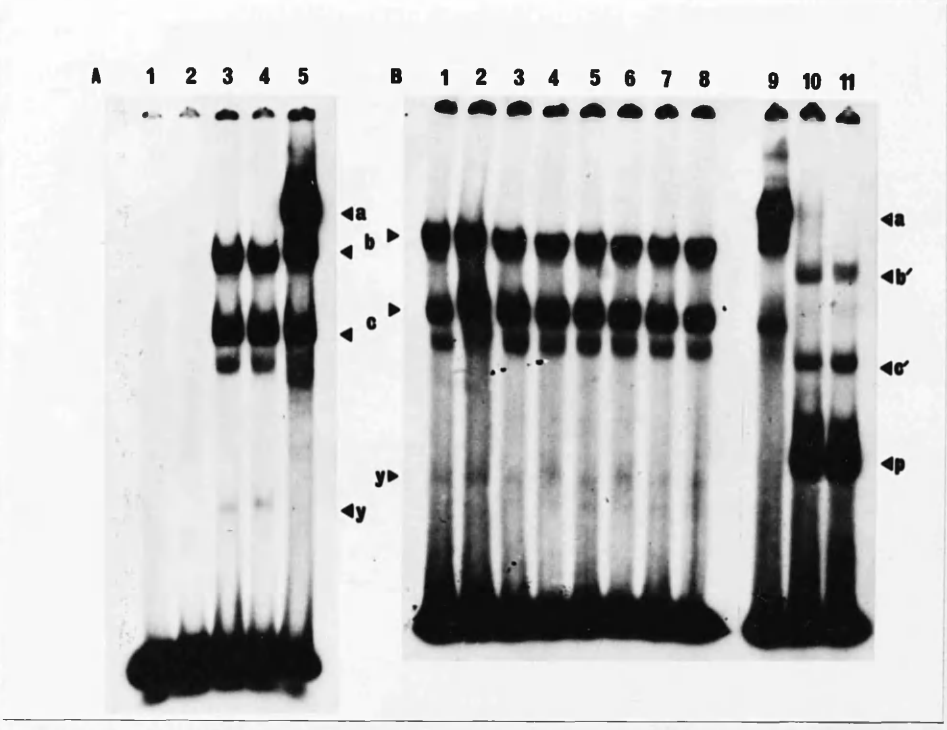


Figure 31: GEM175 protein synthesized in vitro fails to bind detectably to DNA. (A) 5ul In vitro translated GEM175 protein was bound to the IE3 cap site probe at 0°C in the presence (3,4) or absence (1,2) of 2ul mock infected nuclear extract and compared with the wild type Vmw175 complex ("a") in lane 5. No obvious Vmw175/DNA complexes were formed, even with the addition of 2180V antiserum (lanes 2&4). A faint novel complex "y" was further investigated in (B). (B) Reactions 1-8 contain 2ul mock infected extract with in addition 5ul of in vitro translation mix of (1,2) nothing (3,4) pGEM175 (5,6) rabbit reticulocyte lysate alone (7,8) pGEM2 vector. The occurence of "y" was not dependent upon the addition of GEM175 protein, nor was it of similar size to the proteolytic Vmw175/DNA complex "p" in lanes 10 and 11. (Lane 9: 17^{+} infected extract assay as marker, treated with PK in lanes 10 and 11; reactions further incubated with 2180V antiserum in lanes 2,4,6,8 and 11.)

interfering with DNA binding in a similar fashion to its interference with transcriptional regulation (Section 3C3). This could be investigated by making other constructs or by linearizing the transcription template, pGEM175, with alternative restriction enzymes which would remove sequences downstream of the presumed DNA binding domain. An alternative explanation for the failure to detect binding would be if the protein expressed in vitro failed to be post-translationally modified correctly.

SECTION 3E: CONSTRUCTION OF RECOMBINANT HSV-1 VIRUSES

1. Strategy

The aim of the work described in this section was to produce stable recombinant viruses from the HSV-1 strain 17 syn^+ in which both copies of the viral wild-type IE gene 3 were replaced with mutated versions of the gene derived from the plasmid-borne insertion and deletion mutants. These could then be used to investigate further the phenotypes of the mutant gene products and to determine whether the properties of the mutant proteins produced in a normal viral infection reflected their activities in the transient transfection assays (Sections 3B-3D). It was hoped that viruses with novel phenotypes might be produced which could separate the two primary functions of Vmw175, transactivation of early promoters and autoregulation. The recombinant viruses would also provide ideal expresssion vectors to produce large quantities of the mutant polypeptides which could then be further characterized at the physical and biochemical level.

The strategy followed to produce these recombinant viruses was to marker rescue an HSV-1 derivative (which had both copies of IE gene 3 inactivated) by cotransfecting viral DNA with linearized plasmid DNA and then to select or screen for virus plaques in which the plasmid-borne copy of the Vmw175 coding-sequence had replaced the inactive viral copy. In order to propagate the parent strain and any non-functional recombinants it would be necessary to maintain the virus on a complementing cell-line which expresses HSV-1 Vmw175. The cell-line chosen was the mixed line M64A (equivalent to M65, Davidson and Stow, 1985) which had been created by cotransfecting BHK tk^- cells with the tk gene and the linked IE3 gene (carried on the *Xho*I-*Hind*III fragment spanning IR_S) and selecting for tk^+ transformants on HAT medium. These were then screened for production of Vmw175 and ability to complement *ts*K at NPT.

A recombinant virus with the majority of the Vmw175 coding sequence deleted from both copies of IE gene 3 was constructed for use as the parent strain in the marker rescue experiments. This would eliminate the possibility of

rescuing a wild type gene by intragenic recombination between virus and plasmid. The only alternative available null mutant, HSV-1 in1411, had been constructed by inserting stop codons near to the 5' end of the gene (Russell et al., 1987b) and might readily be rescued to wild-type. The use of a parent lacking IE gene 3 sequences would also allow a rapid screen for recombinants regaining these sequences by dot blot hybridization of viral DNA with an IE3 probe.

2. Construction of HSV-1 D30EBA, a derivative of HSV-1 in1411 with a large internal deletion in IE gene 3

The strategy for construction of this recombinant is outlined in Figure 32. The parent for this recombination, HSV-1 in1411 (Russell et al., 1987b), had been created by inserting an oligonucleotide linker encoding a translational stop codon in the IE3 reading frame and a novel XbaI restriction site into the PvuII site at base 248 of both copies of the IE gene 3 coding sequence. HSV-1 in1411 viral DNA was cotransfected into M64A cells with plasmid pdelI11 linearized at the unique BamHI site. Plasmid pdelI11 carries a large deletion from bases 248-3806 within the IE3 coding sequence (see Table 5 above). Virus was harvested from the cells and supernatant four days post transfection and then titred on M64A cells; 36 single plaques were picked under agar stained with neutral red indicator. Cell associated viral DNA for each of these plaques was produced by infecting M64A cells in 1ml Linbro wells and harvesting cell released virus after four days whilst extracting DNA from the infected monolayer. These DNAs were digested with BamHI overnight, resolved on 1% agarose gels electrophoresed in L-Buffer, and analysed by Southern blot hybridization, probing with p175 DNA ^{32}P-labelled in vitro. Figure 33A shows the autoradiograph of the initial screening of some of these plaques. Plaque D30 was chosen for further purification as it exhibited submolar deletions of BamHI k (see Table 11). After three further rounds of plaque purification on M64A cells this recombinant proved stable (see Figure 33B) and was grown up as stock of the desired viral deletion HSV-1 D30EBA.

The genomic structure of D30EBA was further analyzed at

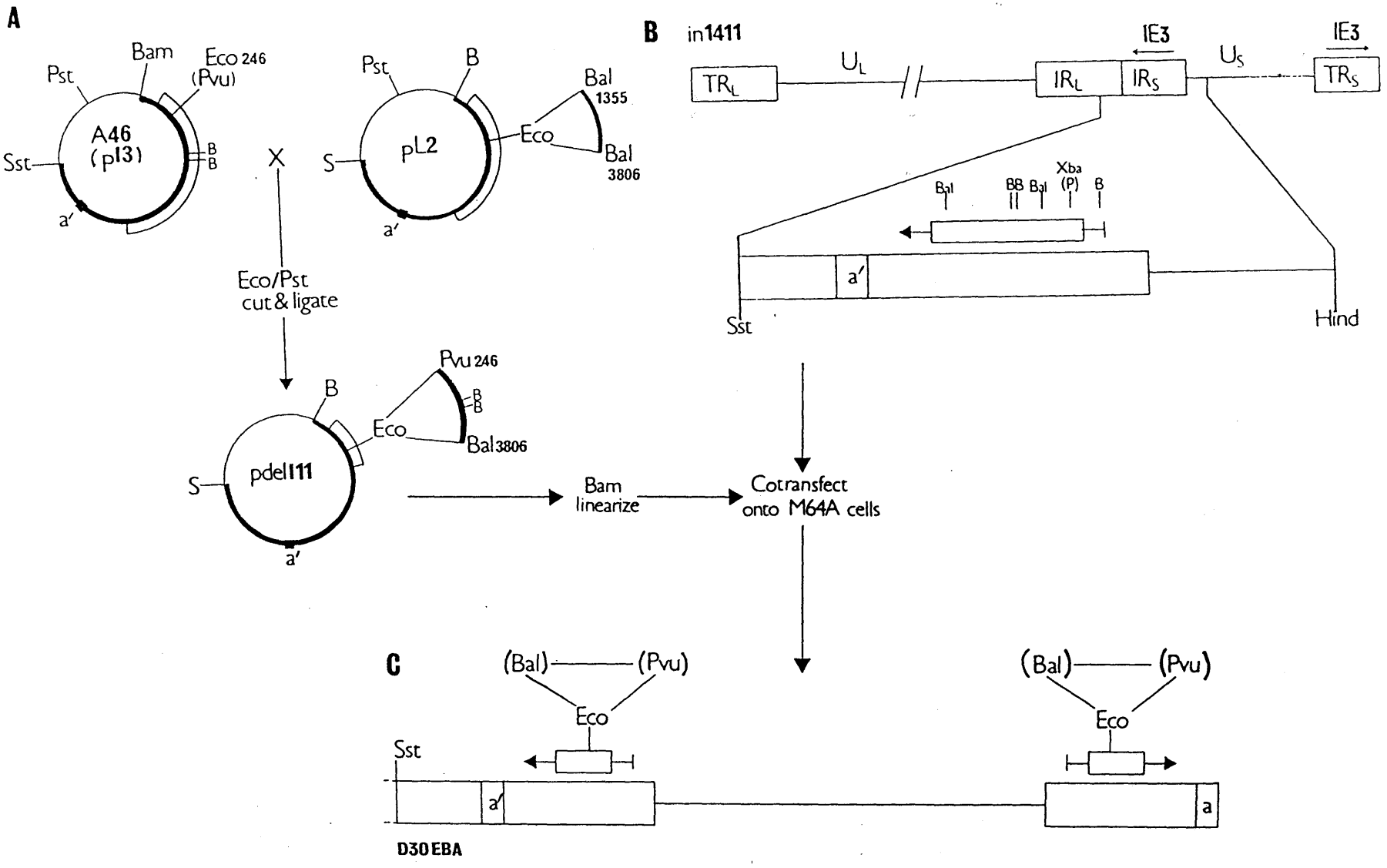

A
Pst
Bam
Eco 246
(Pvu)
A46
(pI3)
Sst
B
B
a'
X
Pst
B
Bal
1355
pL2
Eco
Bal
3806
S
a'
Eco/Pst
cut & ligate
Pvu 246
B
B
B
Eco
Bal 3806
pdel111
S
a'
Bam
linearize
Cotransfect
onto M64A cells
B
in1411
IE3
IE3
U_L
U_S
TR_L
IR_L
IR_S
TR_S
Bal
BB Bal
Xba
(P)
B
a'
Sst
Hind
C
(Bal)
(Pvu)
Eco
Sst
a'
(Bal)
(Pvu)
Eco
a
D30EBA

Figure 32: Construction of the recombinant virus D30EBA carrying a large deletion in both copies of IE gene 3.
(A) Construction of pdelIII. pII3 and pL2 were digested with EcoRI and PstI and recombined in vitro to make the plasmid pdelIII which encodes a copy of IE gene 3 deleted from bases 246-3806 of the ORF. The BamHI sites within the ORF are shown by the capital B. The segments in the arcs show the regions that have been deleted. (B) The structure of the recombinant virus in1411 (Russell et al., 1987b). in1411 contains an XbaI linker insert at base 246 of both copies of the IE gene 3 ORF, which encodes translational stop codons in all three reading frames. This null mutant can express only the first 82 amino acids of Vmw175. (C) Structure of the recombinant virus D30EBA. M64A cells were cotransfected with pdelIII (linearized with BamHI) together with in1411 chromosomal DNA. Recombinant viruses carrying the pdelIII derived deletion in both copies of IE gene 3 were isolated by non-selective screening of picked plaques as described in the text.

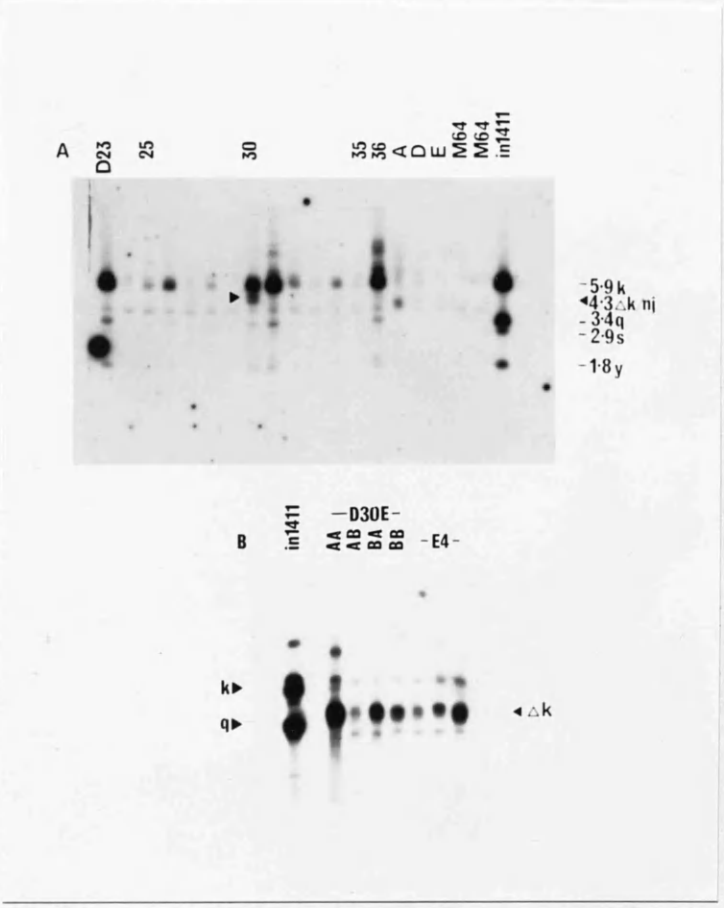


Figure 33: Isolation, screening and purification of the recombinant virus D30EBA. Cell associated DNA was prepared from Linbro wells of M64A cells infected with unselected picked plaques, digested with BamHI and analysed by Southern blot. Filters were hybridized with nick translated pl75, and exposed for autoradiography. (A) Initial screening of plaques picked from cotransfection of pdel111 and in1411 DNA (Figure 32). Parental DNA (in1411) is included as a marker track and fragments spanning the IR_S and TR_S repeat sequences shown to hybridize. A novel submolar 4.3 kb fragment is detected in plaque D30 corresponding to the deleted BamHI-K fragment and forming the new joint fragment (k/nj) in the desired recombinant. Tracks A, D and E are cell associated viral DNA preparations from three different in1411 x pdel111 transformations prior to plaque purification and show no detectable recombinant bands. (B) Viruses carrying this fragment were purified to virtual homogeneity by further rounds of plaque purification (D30EAA, EAB, EBA, EBB). Two clones D30EBA and D30EAB were grown up for further analysis and D30EBA was used as the parent deletion virus in subsequent recombination experiments. (M64: uninfected cellular DNA; E4: independent clone of the deleted recombinant virus isolated from a separate transfection but not purified.)

Table 11: Predicted restriction fragments of recombinant viruses

a EcoRI Fragments

1.	2. 17^+ (& in1411)	3. D30EBA	4. M,O	5. L,N	6. I,J
b=e+k	21.8	17.6/0.6	20.7/1.1	17.3/4.5	20.7/0.8
c=j+k	18.1	13.9/0.6	17/1.1	17.3/4.5	17/0.8
k	5.5	1.3/0.6	4.4/1.1	4.5/1.0	4.4/0.8

b KpnI Fragments

1.	2. 17^+ (& in1411)	3. D30EBA	4. M,O	5. L,N	6. I,J
a=r+j	12.1	8.5	12.1	12.1	11.9
e=r+k	10.6	7.0	10.6	10.6	10.4
j	8.8	5.2	8.8	8.8	8.6
k	6.9	3.3	6.9	6.9	6.7
r	3.7	3.7	3.7	3.7	3.7

c BamHI Fragments

1.	2. 17^+ (& in1411)	3. D30EBA	4. M,O	5. L,N	6. I,J
s	2.9	2.9	2.9	2.9	2.9
q	3.4	nt=1.4	3.4	3.4	3.4
m'	0.2	0.0	0.2	0.2	0.2
y	1.8	0.0	1.8	1.8	1.6
k=s+q	5.9	nj=4.3	5.9	5.9	5.9

d HindIII Fragments

1.	2. 17^+ (& in1411)	3. D30EBA	4. M,O	5. L,N	6. I,J
m	7.5	3.9	7.5	7.5	7.3
g	4.9	1.3	4.9	4.9	4.7
b=d+g	32.1	28.5	32.1	32.1	31.9
c=d+m	34.3	30.7	34.3	34.3	34.1
e=i+g	17.6	14.0	17.6	17.6	17.4
f=i+m	19.8	16.2	19.8	19.8	19.6

Table 11: Tables a-d give the predicted sizes in kb of restriction fragments spanning the IE3 loci based on the sequence data of McGeoch et al., (1988b). Only those fragments which will hybridize to a pl75 DNA probe are listed. Column 1 lists the commonly used fragment names, and columns 2-6 list the predicted fragment sizes for wt HSV-1 (17^+), the deletion D30EBA, and recombinant viruses from the transfections I,J,L,M,N and O, as detailed in Table 12. nt= new terminal fragment, nj= new joint fragment caused by the deletion spanning q,m' and y.

this stage by labelling viral DNA *in vivo* with ^{32}P-orthophosphate (the technique of Lonsdale, 1979) and performing restriction enzyme analysis. Figure 34 shows the autoradiographs of dried down agarose gels of restricted D30EBA DNA compared with that of the parent *in*l411 and wild-type strain 17$^+$. The predicted restriction map of HSV-1 strain 17$^+$ (and *in*l411) for each of the four enzymes *Bam*HI, *Eco*RI, *Hind*III and *Kpn*I is shown in Figure 35; only the regions of the genome adjacent to IE gene 3 are detailed. The size of these restriction fragments is given in Table 11, which also lists the predicted sizes of the new fragments generated by recombination of the pdelI11 deletion into both copies of IE gene 3.

The clone of 17$^+$ used in these analyses clearly has an abnormality in its restriction profile in the region of the short repeat sequences. In all the restriction enzyme digests this virus lacks fragments spanning the *a* sequence or including sequences 3' to IE gene 3 (*Bam*HI-k, *Eco*RI-k, and *Kpn*I-j and k). Further abnormalities are apparent in the *Bam*HI-x, y and z fragments. Fortunately the restriction profile of *in*l411, the parent of D30, appears to be as predicted (Davison, 1981). These abnormalities in the 17$^+$ stock are probably because the stocks were derived from preparations which had not been plaque purified.

The 3.6kb deletion in both copies of IE gene 3 in D30EAB and EBA causes deletions in the joint fragments spanning the long and short segments of the genome and in the short terminal fragments. The predicted fragments have been lost in each case; *Bam*HI k and y (and possibly q), *Hind*III terminal fragments f and g and the internal m fragment, *Eco*RI terminal k fragment, and *Kpn*I terminal fragments j and k. However, there is less obvious evidence of new terminal or internal fragments created in the recombinant genomes by the large deletion and acquisition of an *Eco*RI site (see Table 11). A novel *Bam*HI fragment between n and o is generated, of approximately the expected size (4.3kb), and one new terminal *Kpn*I fragment of 16kb is detected, as is the deleted *Kpn*I m fragment of about 2.5kb. Both of these novel *Kpn*I fragments are arrowed.

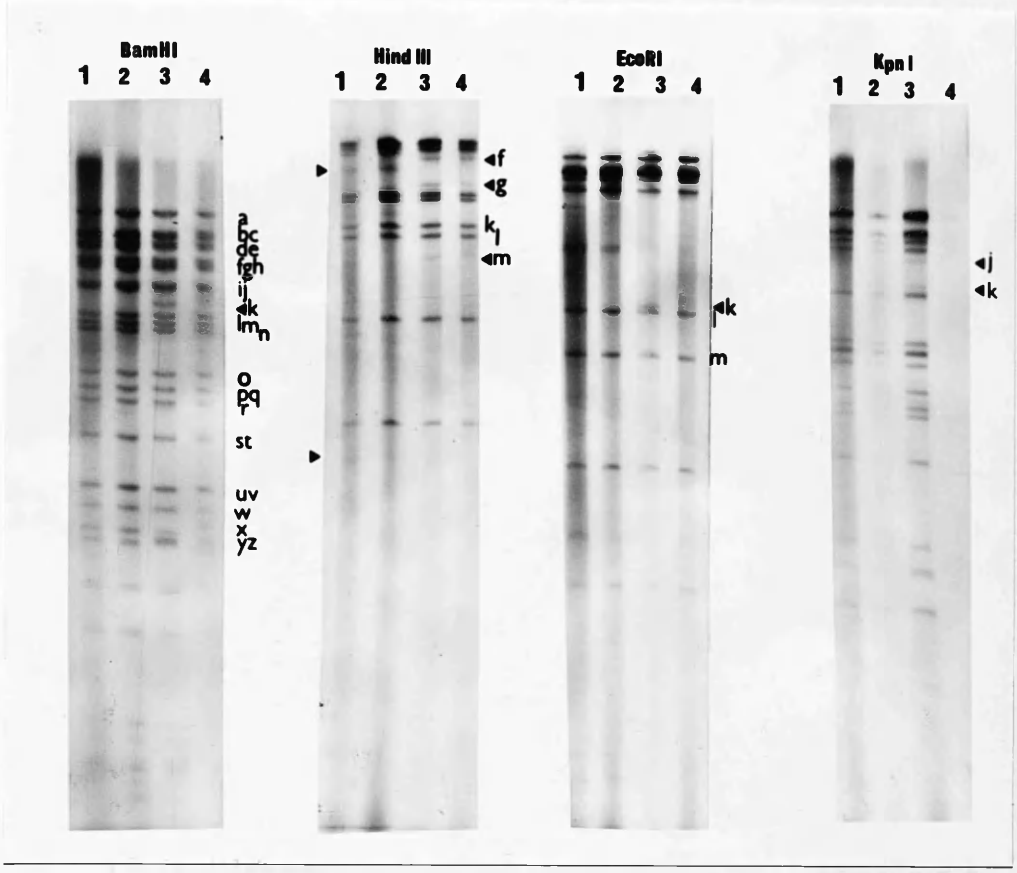
As can clearly be seen with Southern blots of *Eco*RI

Figure 34: Restriction analysis of in vivo labelled D30EBA DNA. ^{32}P-labelled viral DNA was prepared by the method of Lonsdale (1979) and restriction digests analyzed on agarose gels which were dried down before autoradiography. Lane 1: D30EAB, 2: D30EBA, 3: in1411, 4: HSV-1 strain 17 syn$^+$.

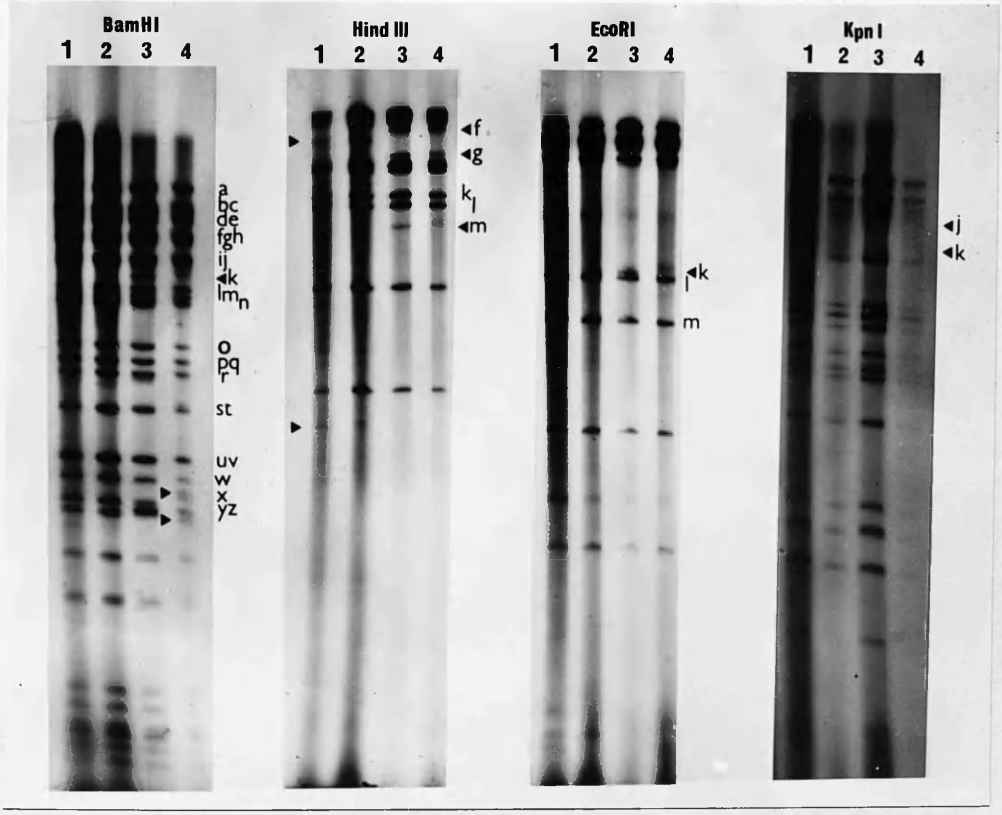
The clone of 17$^+$ used in these analyses clearly has an abnormality in its restriction profile in the region of the short repeat sequences. In all the restriction enzyme digests this virus lacks fragments spanning the a sequence or including sequences 3' to IE gene 3 (BamHI-k, EcoRI-k, and KpnI-j and k, see Figure 35). Further abnormalities are apparent in the BamHI-x, y and z fragments. Fortunately the restriction profile of in1411, the parent of D30, appears to be as predicted (Davison, 1981).

The 3.6kb deletion in both copies of IE gene 3 in D30EAB and EBA causes deletions in the joint fragments spanning the long and short segments of the genome and in the short terminal fragments (see Figure 35). The predicted fragments have been lost in each case; BamHI k and y (and possibly q), HindIII terminal fragments f and g and the internal m fragment, EcoRI terminal k fragment, and KpnI terminal fragments j and k. However, there is less obvious evidence of new terminal or internal fragments created in the recombinant genomes by the large deletion and acquisition of an EcoRI site (see Table 11). A novel BamHI fragment between n and o is generated, of approximately the expected size (4.3kb), and one new terminal KpnI fragment of 16kb is detected, as is the deleted KpnI m fragment of about 2.5kb. Both of these novel KpnI fragments are arrowed.

SHORT EXPOSURE



LONG EXPOSURE



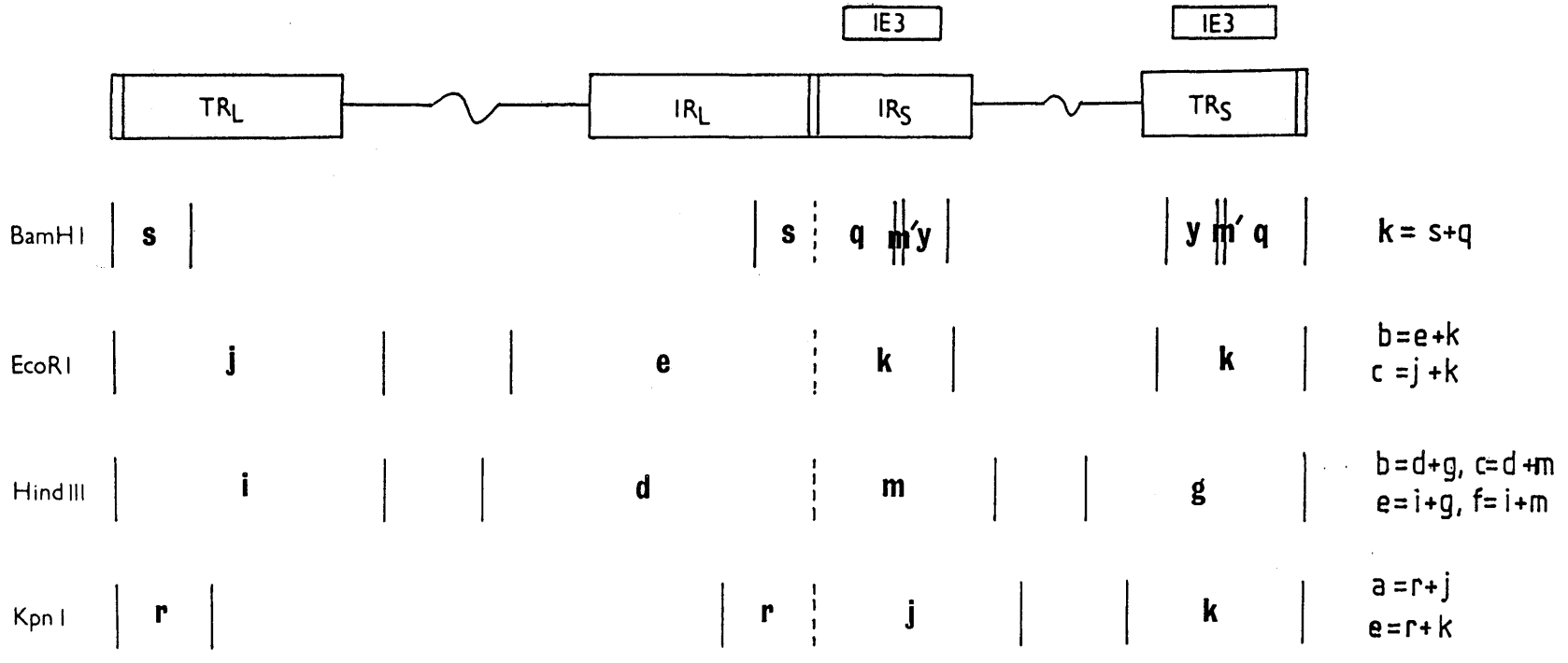


Figure 35: Partial restriction map of HSV-1 strain 17^{+}. The BamHI, EcoRI, HindIII and KpnI restriction maps of the HSV-1 genome in the regions adjacent to IE gene 3 (ie. the repeat sequences) is shown, based on the sequencing data of McGeoch et al., (1988b). Fragments are named according to their mobility on agarose gels (Davison, 1981), only those which potentially hybridize to pl75 sequences are shown. The composition of the joint sequences is detailed. The genome is shown on the prototype orientation (see Figure 1) and the position of IE gene 3 marked.

digested D30EBA (see for example Figures 37 and 39) there is variability in the size of the two sub-EcoRI k fragments, which are predicted to be 1.3 and 0.6 kb in size (Table 11). The multiple bands are probably due to rearrangements and deletions of the 1.3kb fragment containing the G+C rich tandem repeats downstream of IE gene 3, a phenomenon also observed with the cloned plasmid borne IE gene 3 (Section 3A2). These sequences also appear to hybridize very strongly to the probe DNA, presumably due to their great redundancy.

The ability of this deletion virus to be marker rescued to wild-type with the XhoIC fragment (spanning IE gene 3) provides further evidence that no gross secondary mutations have occured in the construction of D30EBA (see Section 3F3).

3. Construction HSV-1 derivatives carrying insertion and deletion mutations in both copies of IE gene 3.

It was originally planned to create a large panel of recombinant viruses by rescuing the large deletion in the genome of D30EBA with a variety of the pl75 insertion and deletion mutants whose construction is described above (Section 3A). Many of these recombinants would be expected to exhibit reduced viability on BHK cells so it was planned to carry out the recombination, screening and purification on the non-selective, complementing cell-line M64A. At the same time a number of recombinations and purifications were performed on wild-type BHK cells which would select for growth of only those mutants producing proteins with sufficient activity to allow virus proliferation. Table 12 summarizes the recombinations carried out and the success or failure of each. Unfortunately no stable recombinants were ever purified on the complementing cell-line M64A. This seems to have been due to the nature of the cell-line, and its growth properties. Not only are plaques slow growing and difficult to detect, except under agar stained with neutral red dye, but the cell-line also appears periodically to lose complementing activity, even when maintained under HAT selection. Furthermore, the presence of multiple copies of IE gene 3 integrated into the genome of these cells

allows recombination of the parental or daughter viruses with these wild-type copies of the gene in addition to the desired viral-plasmid recombinations. Indeed stocks of both in1411 and D30EBA carry about 0.001-0.01% wild-type contaminants, presumably due to this phenomenon, and six apparently wild-type viruses (P1-6) were picked after D30EBA DNA alone had been transfected onto M64A cells (see Table 12 and restriction profiles in Figure 36).

The main obstacle to success in constructing a panel of mutant viruses therefore seemed to be one of spurious recombination; consequently plaques picked on M64A cells, which had initially seemed to exhibit the desired recombination were found to rearrange on further passage. Similar to the problems experienced with the propagation of plasmid p175 in E.coli (discussed earlier, Section 3A2) many of these rearrangements appeared to be in the region downstream of the IE3 coding sequence in a highly repetitive region of DNA. The problems encountered are illustrated by following the purification of one recombinant I15HBC. The initial recombination was carried out on M64A cells and recombinant clones were clearly detectable after the first plaque purification (Figure 37A). On further purifications, however, variability in the size of fragments ranging between 0.5 and 1kb became apparent (Figure 37C) this only stabilized when purification of this virus was switched to BHK cells (Figure 37D). The 5' EcoRI k subfragment, predicted to be 0.8kb in size, should be invariant, and is probably the (arrowed) band running just above the D30EBA sub-k band of 0.6kb (Figure 37C and E).

The results of attempts to recombine in several mutant IE3 alleles are detailed in Table 12. Only three new viruses were recovered which carry the desired mutation: O2/O9, N9/N10 and I15. O2 and O9, and N9 and N10, were independently picked plaques from the same transfection. The structures of these three viruses are shown in Figure 38 and described in Table 13.

Figure 39 shows a representative Southern blot of EcoRI digested viral DNA prepared from infected Linbro wells and hybridized to radiolabelled p175 DNA. Plaque purified isolates of the N and O recombinant insertion mutants are

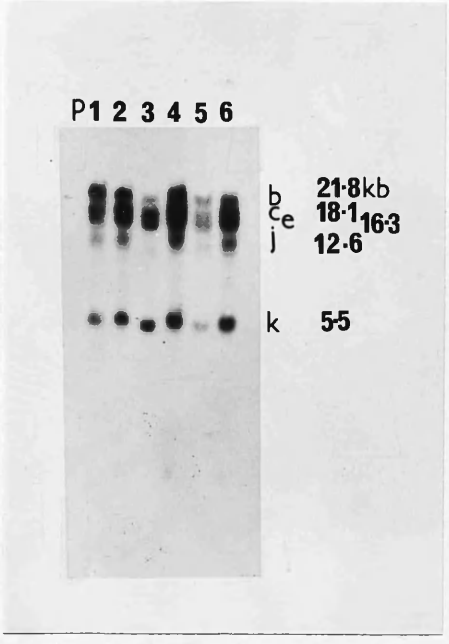


Figure 36: Analysis of spontaneous revertants of the virus D30EBA. Six plaques (P1-6) isolated from the transfection of D30EBA onto non-complementing BHK cells were purified and cell associated DNA was prepared from BHK cells infected in Linbro wells. An autoradiograph of a Southern blot analysis performed on EcoRI digested DNA is shown, probed with primer extended p175. Each of the clones has regained fragments of the genome which are absent in the parent D30EBA, in particular note the reappearance of the EcoRI-k fragment which spans the entire IE3 gene. (Fragments were identified relative to in1411 and D30EBA control digestions, not shown). These wild-type revertants of D30EBA were probably created by recombination between the viral chromosome and the integrated chromosomal copy of IE gene 3 in the complementing M64A cells.

Table 12: Plasmid-virus recombination by marker rescue of HSV-1 D30EBA

1. EXPT.	2. PLASMID	3. CELLS	4. TITRE	5. NUMBER OF POSSIBLE RECOMBINANTS	6. CLONES PURIFIED	7. FATE
F	R10 (pI16)	M64A	2.10^4(M64A)	a 4/18 b 0/48	none	
G	R74 (pI29)	M64A	2.10^4(M64A)	a 3/16 b 1/44	G4	unstable
H	R9 (pD9)	M64A	3.10^3(M64A)	a 2/16 b 0/16	none	
I	D10 (pD1)	M64A	1.10^4(M64A)	a 4/16 b 1/16	I15	I15HBC stable on BHK
J	D10 (pD1)	M64A	nd	4/13	J11,J12	unstable
K	R2A (pI12)	M64A	nd	nil	none	
L	S61 (pI39)	M64A	nd	nil	none	
M	S24R4 (pI9)	M64A	nd	3/13	M1,M4,M5	unstable
N	S61 (pI39)	BHK	2.10^5	15/16	N9,N10	N9BAA,N10DAA stable
O	S24R4 (pI9)	BHK	1.10^7	15/16	O2,O9	O2AAA,O9CBA stable
P	nil	BHK	6.10^2	6/6 (wt)	P1-6	
Q	R9 (pD9)	M64A	nd	nil	none	
R	R2A (pI12)	M64A	nd	nil	none	
S	R10 (pI16)	M64A	nd	8/48	S2,8,12,16, 18,28,36,46	none correct
T	R74 (pI29)	M64A	nd	7/24	T5,8,16	unstable
U	R10 (pI16)	M64A	5.10^5	not screened		

Table 12: For each transfection experiment (F-U, column 1) the parental plasmid linearized with KpnI and recombined with D30EBA DNA is given in column 2; the original construct name and published name (in parentheses) are shown (see Tables 4 and 5). The cell type used for the initial transfection and plaque purification is given in column 3, and the titre of pfu/ml recovered in column 4. Column 5 records the number of potential recombinants detected by Southern blot analysis (or for transfections F-I; a from dot blot analysis, b from Southern analysis). Plaques purified are listed in column 6, and the fate of these clones recorded in column 7.

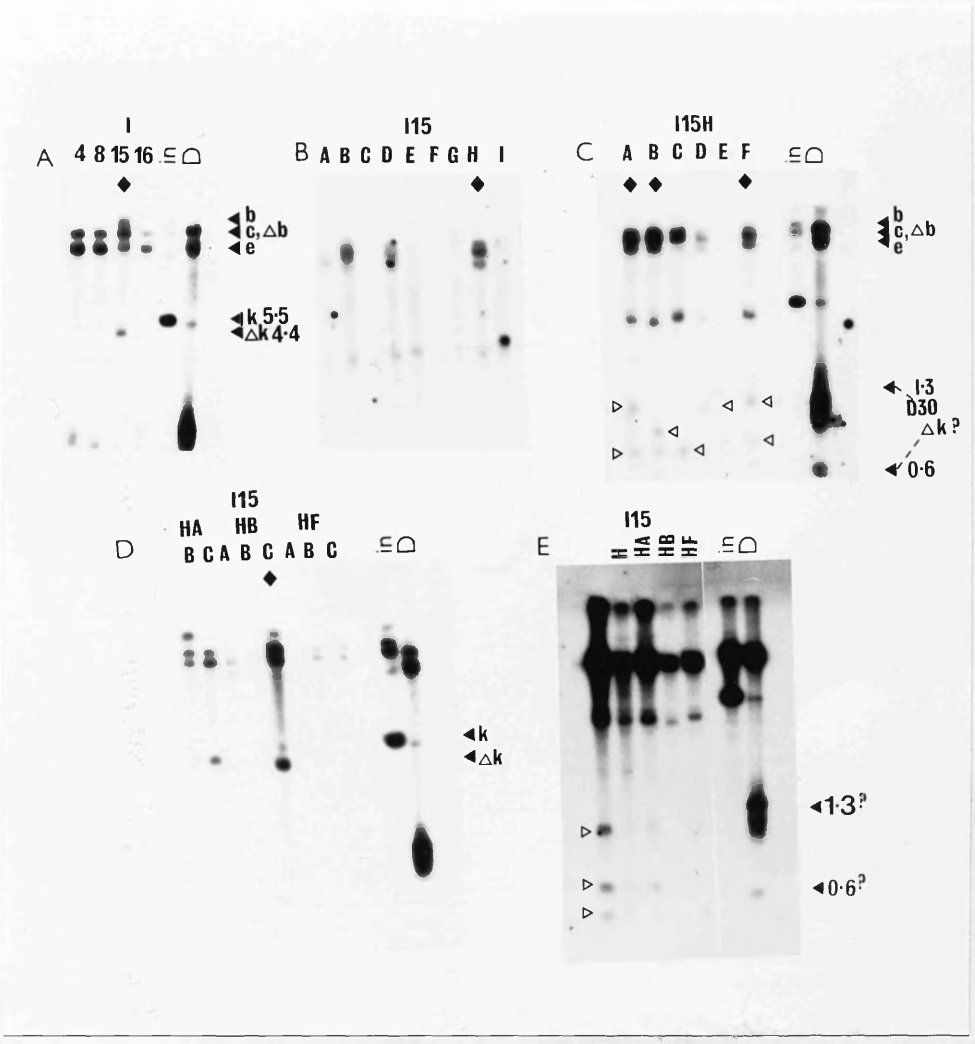
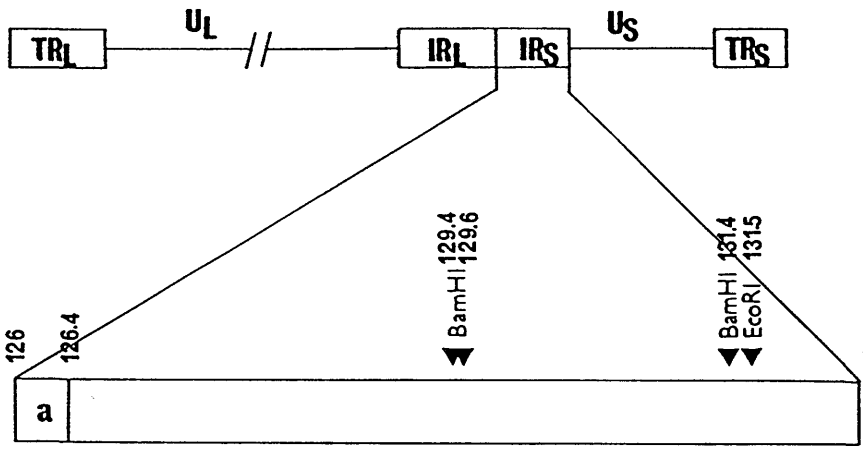
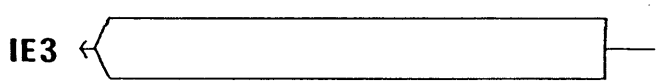


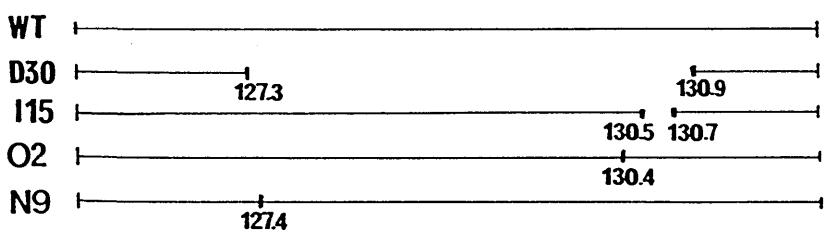
Figure 37: Isolation, screening and purification of the recombinant virus I15HBC. Successive stages in the plaque purification of the recombinant virus I15HBC are shown as autoradiographs of Southern blots on EcoRI digests of cell associated virus DNA. In all but the final purification viruses were propagated on M64A cells. (A) Initial screening of plaques chosen as potential positives on the basis of a dot blot hybridization experiment (results not presented). Tracks of parental D30EBA ('D') and in1411 ('in') DNA are included as markers. I15 is the only clone to regain (deleted) EcoRI k and b fragments (see Figure 35 and Table 11 for restriction maps). (B) Second plaque purification of I15. Clone H was selected as carrying the deleted EcoRI b and k fragments. (C) Third plaque purification of I15. A number of clones stably maintaining the 4.4kb deleted EcoRI k band are shown, however, the smaller (arrowed) EcoRI fragments (possibly including the other deleted sub-k fragment of 0.8kb) were found to vary in size between isolates. (D) The final plaque purification was performed on non-complementing BHK cells. The clone I15HBC was grown up for further characterization. (E) Comparison of various stages in plaque purification analyzed together on a single blot. The variable sub-k fragments are arrowed.

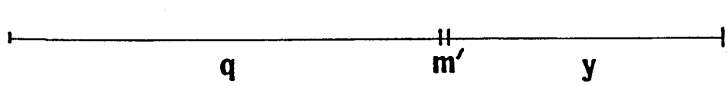
A





EcoRI-k





WT BamHI fragments

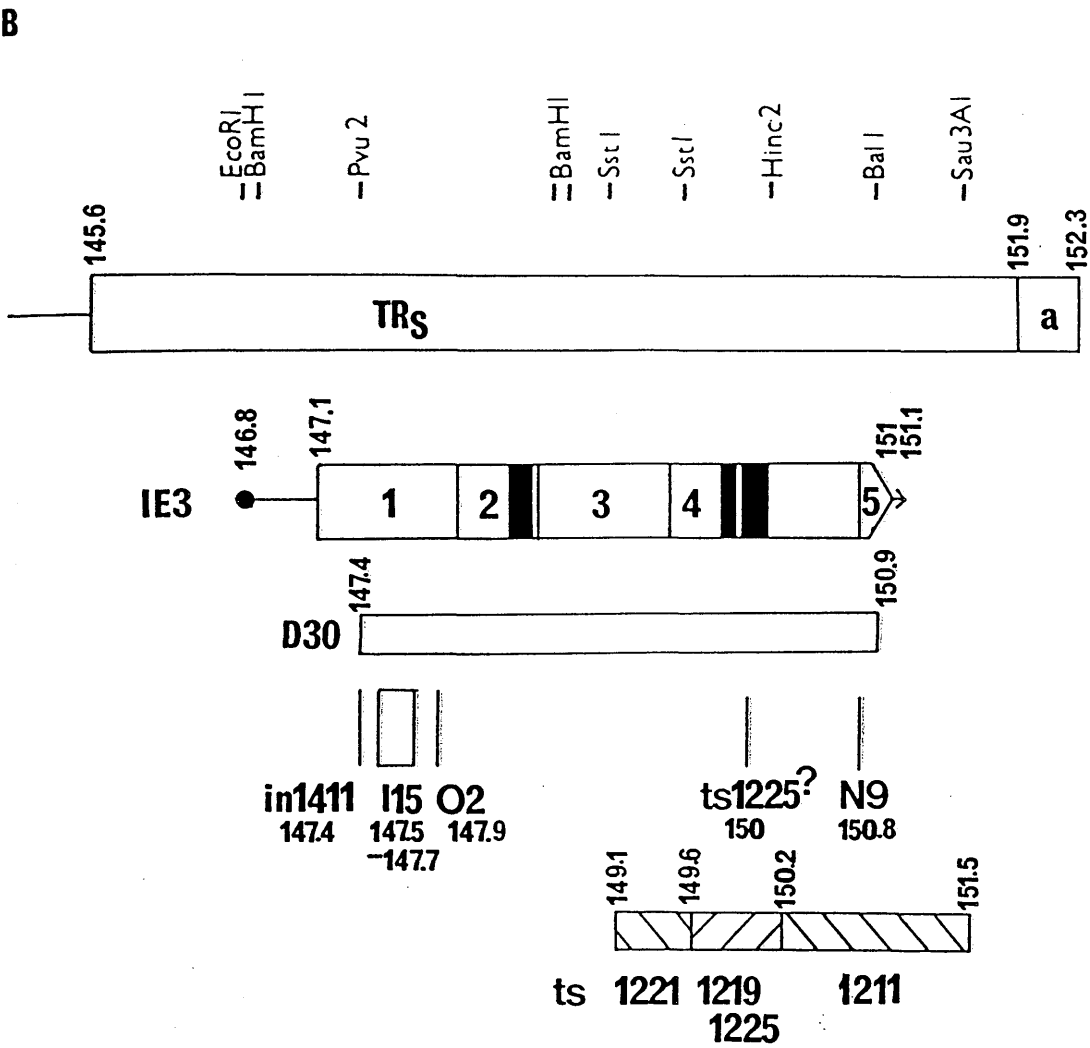


Figure 38: Structure and map coordinates of the DNA lesions of the recombinant and temperature sensitive mutants within the viral IE gene 3 locus. (A) The structures of the recombinant viruses D30EBA, I15HBC, O2 (& O9) and N9 (& N10) are shown. The extent of the novel deletions and the position of the inserted EcoRI linkers are shown for the copy of IE gene 3 present in IR_S. The coordinates are given in kb. The size of the novel EcoRI k fragments generated in each recombinant are also given, and the position of BamHI sites also marked. (B) The structures of the recombinant and ts viruses are shown within TR_S with the appropriate coordinates given in kb. The IE gene 3 reading frame is divided into the five homology regions as shown in Figure 5. The map coordinates for fragments capable of marker rescuing the four ts mutations are given (provisional data of Drs. V.Preston and R.D.Everett, per.comm.). The lesion in ts1225 has provisionally been mapped to a single base substitution at 150.0kb, within the most conserved portion of region 4 (R.D.Everett, per.comm.).

Table 13: Structure of recombinant viruses: co-ordinates of insertion and deletion mutants

1. virus	2. expt.	3. plasmid name	4. plasmid co-ords.	5. IR_S co-ords.	6. TR_S co-ords.
D30EBA		pdelI11	250-3808	127.3-130.9	147.3-150.9
I15HBC	I,J	D10 (D1)	483-686	130.5-130.7	147.5-147.7
02/09	M,O	S24 (I9)	752	130.4	147.9
-	K,R	R2A (I12)	927	130	148
-	U,S,F	R10 (I16)	1116	129.8	148.2
-	T,G	R74 (I29)	2799	128.3	149.9
-	H,Q	R9 (D9)	2801-3292	127.8-128.3	149.4-150.4
N9/N10	L,N	S61 (I39)	3715	127.4	150.8

Table 13: For each recombination experiment (F-U, column 2, see Table 12) the co-ordinates of the site of mutation in the parental plasmid are given in column 4. These co-ordinates refer to the bases of the Vmw175 coding-sequence and show the extent of deleted bases or the co-ordinate of the site of linker insertion (see Tables 4 and 5). The co-ordinates of the mutation sites within each copy of IE gene 3 in IR_S and TR_S (columns 5 and 6) are based on the complete nucleotide sequence of HSV-1 strain 17^+ (McGeoch et al., 1988b). The parental plasmids used in the recombinations are given their original construct name, and their published name in parenthesis (column 3).

shown (O2AA, O9CB, N9BA and N10DA). These have regained IE3 sequences absent in the D30EBA parent, which are cleaved at the inserted EcoRI linker. The O2/O9 genome has gained two new sub-EcoRI k subfragments of 4.4 and 1.1 kb (see Table 11) and the N9/N10 genome has gained two new sub-EcoRI k subfragments of 4.5 and 1.0 kb. The 1.0 kb fragment differs slightly in size between N9 and N10, probably reflecting rearrangements in the repeat sequences downstream of IE gene 3. Examples of picked plaques from other transfections (I, J, M, S, and G, see Table 12) are also shown. Clearly many of these clones have regained IE3 sequences not present in D30EBA, but the new fragments rearranged on serial plaque purification and did not readily correspond with the predicted recombinant fragment sizes.

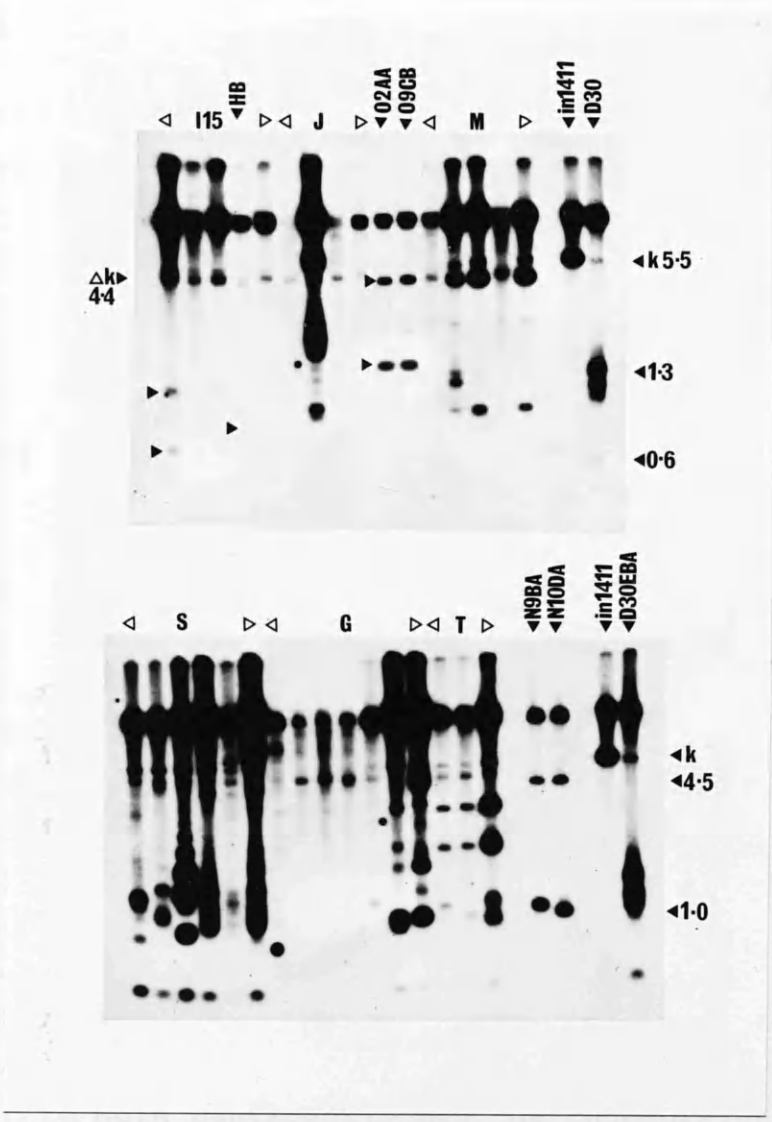


Figure 39: Isolation and screening of recombinant viruses carrying lesions within IE gene 3. Southern blot analysis of viral DNA digested with EcoRI, resolved on a 1.0% agarose gel, blotted and probed with primer extended p175 probe. in1411 and D30EBA controls are included to show the size of the wild type EcoRI k fragment deleted in the parent virus (D30EBA). The novel EcoRI subfragments regained in the 02/09 and N9/N10 recombinants are highlighted (4.4 & 1.1, and 4.5 & 1.0kb respectively). The new 1.0kb band in the N9 and N10 recombinants may vary slightly in size due to rearrangements in the sequences downstream from IE gene 3. Also shown are several other virus plaque isolates with recombinant genomes isolated from early passages of recombination transfections, but unstable on further passage (I15: see Figure 37; J,M,S,G,T: see Table 12). All viral DNA is prepared as cell associated except in the cases of N9/N10, in1411 and D30EBA where DNA was prepared from cell released virus particles. Faint variable bands in I15 plaques are highlighted.

SECTION 3F: CHARACTERIZATION OF RECOMBINANT HSV-1 VIRUSES

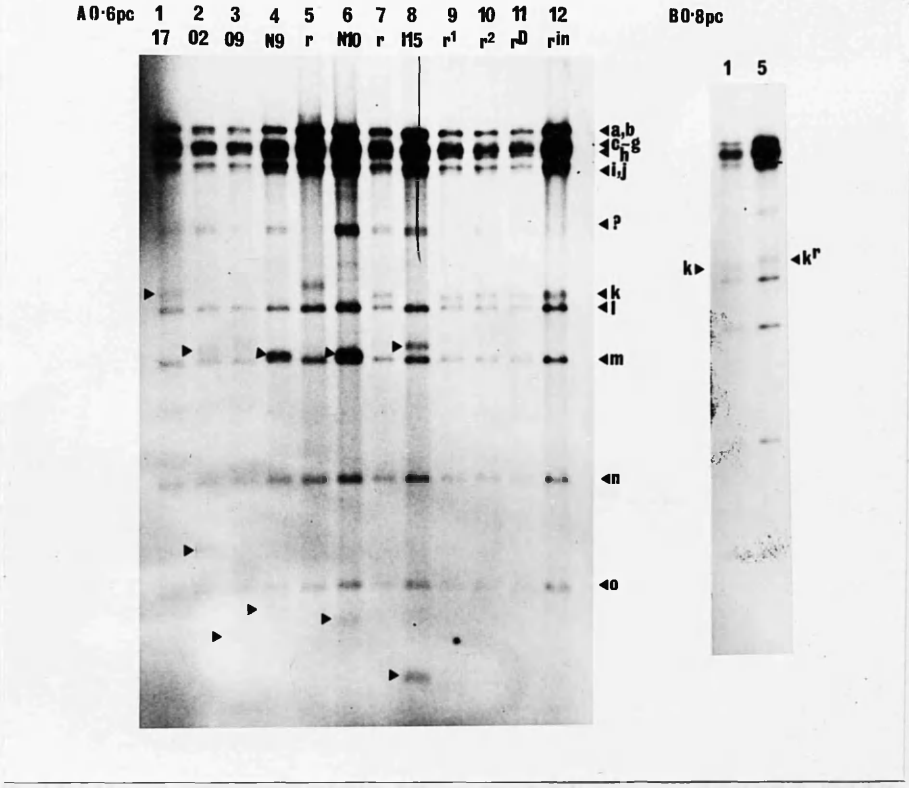
The basic growth, replication and Vmw175 DNA binding properties were characterized for each of the recombinant viruses D30EBA, I15HBC, O2/O9 and N9/N10. In addition five viral temperature sensitive mutants with lesions in IE gene 3 were characterized. Figure 38 shows the positions mapped for the lesions present in these five viruses; ts1211, 1219, 1221, 1223 and 1225, derived from marker rescue data of V.G.Preston and R.D.Everett (per. comm.).

1. DNA Structure

Each of the three recombinant viruses were purified on BHK cells after initial characterization by Southern blot analysis of cell associated DNA (Section 3E3). The predicted sizes of restriction fragments spanning the IE3 locus are given in Table 1 1. The co-ordinates of the inserted EcoRI site should be readily verifiable by simple restriction enzyme mapping; however, the occurence of deletions 3' to IE gene 3 in the plasmid clones (as discussed above, Section 3A2) and possible variability in the number of a sequences in recombinant viruses, might lead to anomalies in the observed fragment sizes of recombinants. In order to more confidently map the recombinant genomes, ^{32}P-labelled viral DNA was prepared and analyzed by restriction mapping with EcoRI (Figure 40). In each of the recombinant genomes the terminal EcoRI k fragment spanning IE gene 3 has been cut. This band is regained in wild-type rescued recombinants of N9, N10 and I15 (see Section 3F3). The quality of the gels does not allow complete verification of the novel subfragments generated in each recombinant. The I15 k band is cleaved to give the expected subfragments of 4.4 and 0.8kb (Table 11). The larger 4.4 and 4.5 kb O and N subfragments are detected (the O fragment would appear to be somewhat larger than predicted, but carries the potentially variable sequences 3' to IE3) but the smaller bands are not unambiguously detected. Possible bands are highlighted, but these bands were well characterized by Southern blot analysis (Section 3E3, Figure 39).

Within the limits of these restriction analyses, caused

Figure 40: Restriction analysis of the in vivo labelled genomes of the recombinant viruses and their marker rescued revertants. ^{32}P-labelled viral DNA was prepared by the method of Lonsdale (1979) and EcoRI restriction enzyme digests analyzed on 0.6% and 0.8% (pc) agarose gels which were dried down before autoradiography. The EcoRI k fragment is absent or cleaved in lanes 2 (O2AAA), 3 (O9AAA), 4 (N9BAA), 6 (N10DAA) and 8 (I15HBC); but regained in viruses marker rescued from these mutants, lane 5 (N9^{r}), 7 (N10^{r}), 9 & 10 (I15^{r}), 11 (D30EBA^{r}) and 12 (in1411^{r}). Rescue in each case was performed with the XhoIC fragment, excepting I15^{r2} which was rescued with a fragment internal to IE gene 3 (see Table 15). Note that the rescued k fragment in N9^{r} is larger than in other viruses, and that as previously discussed (Figure 34) the k fragment is under represented in this clone of HSV-1 strain 17^{+} (lane 1). The novel, recombinant sub-k fragments are arrowed in the recombinant viruses and seem where visible to be of the expected sizes (see Table 11 and text). However, in this analysis, unlike the Southern blot above (39), the 1.1kb 5' O2/O9 EcoRI-k subfragment may differ in size between the O2 and O9 isolates, although this fragment is not clearly distinguishable. The variation in the 3' N9/N10 EcoRI-k subfragment was seen on the Southern blot and may reflect heterogeneity in sequences downstream of the IE3 coding sequence. The band labelled "?" is of unknown origin and frequently observed in HSV-1 EcoRI digests (Davison, 1981).


A0·6pc
1 17
2 02
3 09
4 N9
5 r
6 N10
7 r
8 H5
9 r1
10 r2
11 rD
12 rin
a,b
c
g
h
i,j
P
k
l
m
n
o
B0·8pc
1
5
k
kr

primarily by the obvious variability in the sequences downstream of IE gene 3, the structure of all three recombinant viruses is as predicted in Table 11. This is supported by the ability of (at least two of) these viruses to be rescued by the XhoIC fragment spanning IE gene 3 (Section 3F3). Only by subcloning and sequencing the mutant IE3 genes could their structure be unequivocally verified.

2. Growth properties and temperature sensitivity

The three viruses constructed from the parent D30EBA are all viable at 37°C on BHK (non-complementing) cells. However, as recorded in Table 14, each virus exhibits a degree of temperature sensitivity and I15HBC has a temperature dependent syncitial (syn^-) plaque morphology. The syncitial plaques formed by I15HBC on BHK cells are smaller and less-defined than wild-type (syn^+) plaques. This phenotype is more marked at higher growth temperatures.

The five mutant virus strains originating from Dr V.G.Preston's laboratory show varying degrees of temperature sensitivity, or "leakiness" on BHK cells. Whilst mutants ts1211, ts1219 and ts1223 are severely restricted for growth at the NPT of 38.5°C, ts1221 and ts1225 plaque well at temperatures of 39°C or below, with 39.5°C being non-permissive, (Table 14).

3. Marker rescue of recombinant viruses

In order to ensure that any phenotypes that the recombinant viruses might exhibit were due to the lesions introduced into IE gene 3 and not caused by a second site mutation it was important to rescue these viruses with a cloned wild-type copy of IE gene 3 and then to verify that these rescued virus now behaved identically to wild-type HSV-1 17 syn^+, the original parent virus.

Marker rescue experiments were carried out on BHK cells by co-transfecting viral DNA and a cloned copy of the HSV-1 17^+ derived XhoI C fragment which spans IE gene 3 (linearized plasmid pGX58). As detailed in Figure 40 and Table 15 this XhoI C fragment rescued both of the null mutant parents in1411 and D30EBA to wild-type, and the temperature sensitive viruses N9, N10 and ts1225 were

Table 14: Temperature sensitivity of recombinant viruses

VIRUS	TITRE ON BHK CELLS (pfu/ml) 31°C	37°C	38.5°C	39.5°C
O2AAA (cav)	-	1.10^9	8.10^8	$<10^7$
O9CBA (cav)	-	1.10^9	6.10^8	$<10^7$
N9BAA (cav)	5.10^8	5.10^8	-	$<10^4$
	-	2.10^9	1.10^9	$<10^7$
N10DAA (cav)	4.10^8	5.10^8	-	$<10^4$
	-	5.10^8	2.10^9	$<10^7$
I15HBC (cav)	$2.10^7 syn^{+/-}$	$5.10^7 syn^-$	$3.10^6 syn^-$	-
(crv)	$8.10^7 syn^{+/-}$	$9.10^7 syn^-$	$3.10^7 syn^-$	-
tsl211 (cav)	$1.5.10^9$	-	5.10^5	$<10^4$
tsl219 (cav)	$1.5.10^9$	-	$<10^4$	$<10^4$
tsl221 (cav)	7.10^8	-	6.10^8	$<10^4$
tsl223 (cav)	$1.5.10^9$	-	$<10^4$	$<10^4$
tsl225 (cav)	$1.5.10^9$	-	$1.7.10^8$	$<10^4$

Table 14: Temperature sensitivity of recombinant viruses. Titres given (pfu/ml) were obtained by fixing and staining infected cell monolayers 2 days post infection (3 days at 31°C). The syncitial phenotype of I15HBC is recorded; syn^+ virus forms wt non-syncitial plaques, syn^- plaques are syncitial.

Table 15: Marker Rescue of Recombinant Viruses

Expt.	Virus	Rescuing DNA	Titre (pfu/ml) 31°C	37°C	39.5°C
5	tsl225	none	9.10^4	-	$<1.10^2$
6	tsl225	pGX58	5.10^4	-	3.10^4
7	N9BAA	none	$>5.10^6$	-	$<1.10^2$
8	N9BAA	pGX58	4.10^5	-	1.10^5
9	N10DAA	none	4.10^6	-	$<1.10^2$
10	N10DAA	pGX58	3.10^4	-	2.10^4
1-1	D30EBA	none	-	<10	-
1-2	D30EBA	pGX58	-	3.10^6	-
1-7	in1411	none	-	10	-
1-8	in1411	pGX58	-	6.10^5	-
1-9	I15HBC	none	2.10^6(+)	-	2.10^5(-)
1-10	I15HBC	pGX58	$>5.10^6$	-	3.10^6(+)
2-1	I15HBC	pI24 EcoRI-SalI frag.	3.10^6(+)	-	$2.5.10^4$(+/-)

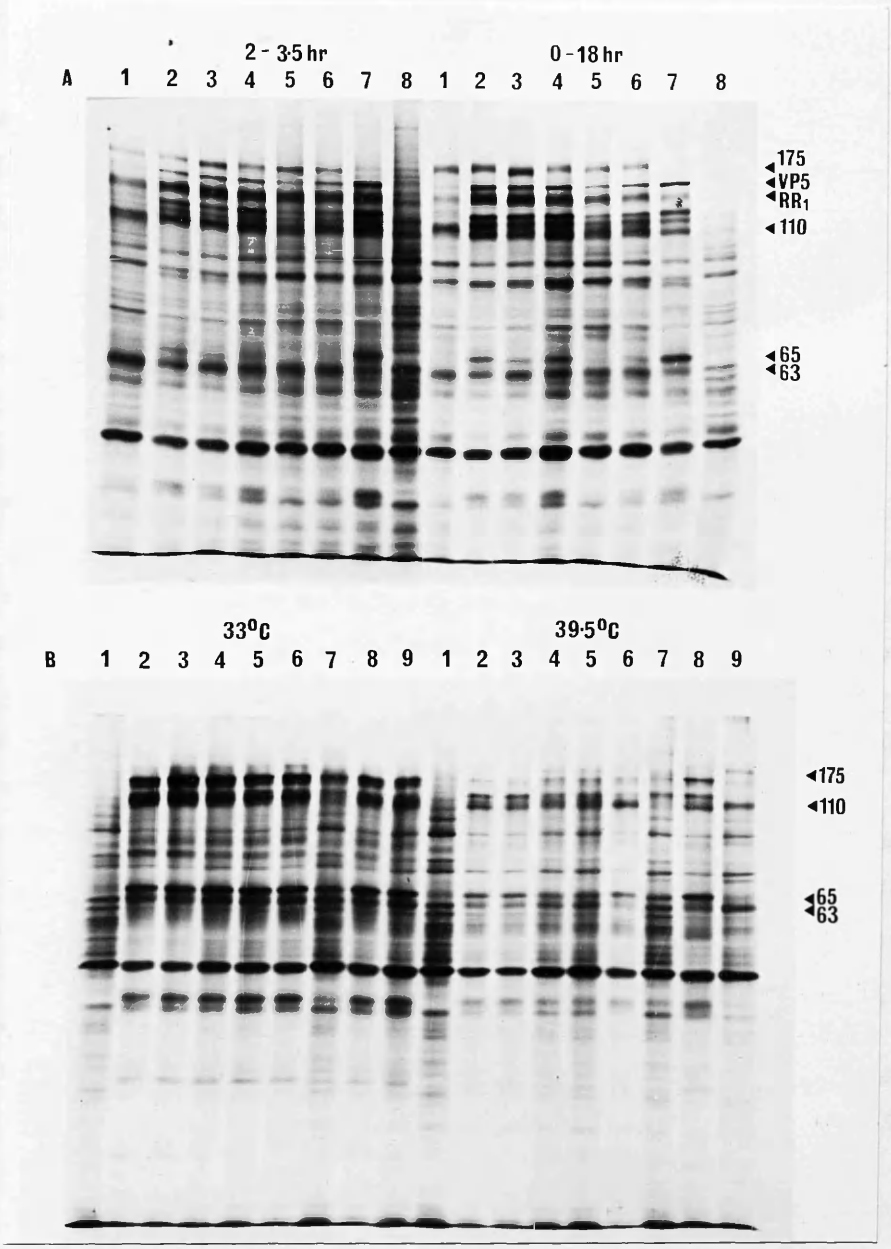
Table 15: BHK cell monolayers were transfected with 0.5ug of cell released viral DNA with or without a ten-fold molar excess of linearized rescuing DNA. pGX58, cut with XhoI, contains the HSV-1 XhoI C fragment (co-ordinates 123.0-133.5); the EcoRI-SalI fragment prepared from pI24 (Table 4) spans bases -129 to +2041 of the IE gene 3 ORF and includes those bases deleted in I15HBC (483-686). The monolayers were harvested after 3 days incubation at 31°C and titred at 31, 37 and 39.5°C. The plaque morphology of experiments 1-9, 1-10 and 2-1 is recorded as + (syn^+) - (syn^-) or +/- (a mixture).

rescued to virus capable of growth at the NPT. The temperature dependent syncitial mutant I15HBC was rescued to wild-type both with the XhoI C fragment and with a 2.2kb fragment from the 5' end of the IE3 ORF (-126 to +2041). This result implies that the unusual plaque morphology of this virus is due to the lesion in IE gene 3 and not some second site mutation. This defines a novel phenotype for viruses producing mutant Vmw175 polypeptide. The small and syncitial plaque phenotype of I15HBC (deleted for amino acids 162-229) is similar to that of a virus described by Schröder et al., (1985) with a spontaneous deletion within the IE3 coding region removing codons 209-236, although it was not clear that the small plaque morphology of their mutant was a direct consequence of this deletion.

4. Gene expression: polypeptide profiles of recombinant viruses

Preliminary experiments were performed to examine the gene expression properties of the recombinant viruses. BHK monolayers infected at a moi of 10 pfu/cell in methionine-free EC5 were labelled overnight with ^{35}S-methionine at 31 or 39.5^{o}C and the protein extracts were resolved on 7.5 or 10% SDS-PAGE gels (Figure 41). The phenotypes of the recombinant viruses were compared to wild-type virus (17^{+}) and the well characterized virus tsK which possesses a "tight" temperature-sensitive mutation in Vmw175, allowing no early or late gene expression at the NPT. At 39.5^{o}C the viruses N9, N10, ts1211, 1219, 1221, 1223 and 1225 all overexpress Vmw175, showing a failure to autoregulate IE gene expression. However, all of these mutants exhibit a different pattern of gene expression to tsK and are "leaky" in the sense that they allow expression of later classes of genes, for example VP5. Whilst some of the viruses are deficient in some late gene products, such as Vmw65 (ts1211 and 1219) others show few obvious deficiencies compared to wild-type. I15HBC seems to grow poorly, and may shut-off host gene expression less efficiently than wild-type (Figure 41). O2 and O9 induce a polypeptide profile similar to that of wild-type at both NPT and PT and do not overexpress Vmw175 at NPT. These

Figure 41: Polypeptide profiles of recombinant and temperature sensitive viruses at permissive and non-permissive temperatures. SDS polyacrylamide gels of ^{35}S-methionine labelled total cellular proteins. The positions of IE proteins Vmw175, 110 and 63 and the early and late proteins RR1, VP5 and Vmw65 are shown. (A) (7.5% Gel) ts-Viruses labelled from 2-3.5h p.a. or 0-18h p.a. at 39.5^{o}C. (Lane 1) tsK, (2) ts1225, (3) ts1223, (4) ts1221, (5) ts1219, (6) ts1211, (7) 17^{+}, (8) mock infected. None of the ts viruses are as restricted in early and late gene expression as tsK. All of the ts viruses overexpress Vmw175 but express late gene products to different extents (for example VP5 and Vmw65). (B) (10% Gel) Recombinant viruses labelled for 1-18h p.a. at 33^{o}C and 39.5^{o}C. (Lane 1) mock, (2) O2, (3) O9, (4) N9, (5) N10, (6) ts1225, (7) I15HBC, (8) 17^{+}, (9) tsK. At 33^{o}C the pattern of polypeptides synthesized by all of the viruses resembles that of WT (17^{+}). The recombinants N9 and N10 overexpress Vmw175 at the NPT, like ts1225 and tsK. Otherwise the polypeptide profiles of the O2/O9 and N9/N10 recombinants more closely resemble that of WT (17^{+}) virus than the tsK control. The O2 and O9 recombinants autoregulate Vmw175 production at NPT. The virus I15 appears to shut off host polypeptide synthesis at 39.5^{o}C inefficiently.


A
2 - 3·5 hr
0 - 18 hr
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
175
VP5
RR1
110
65
63
B
33°C
39·5°C
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
175
110
65
63

preliminary results should be confirmed and investigated in more detail.

5. DNA replication by recombinant viruses

The ability of the recombinant viruses to replicate viral DNA was determined at the permissive and non-permissive temperatures. BHK cells were infected at a moi of 10pfu/ml in Linbro wells and cell associated DNA prepared 0 or 16 hours post infection at PT or NPT. Levels of viral DNA recovered were measured by slot blot hybridization using as a probe pGX156 which carries the viral EcoRI H fragment spanning U_S. The results are presented in Figure 42.

All the viruses assayed are replication competent at 33°C. However, I15HBC replicates less efficiently than wild-type in that the level of input DNA is far higher, probably reflecting the low titre and poor growth of this virus and indicating that it probably has a high particle to infectious pfu ratio. Furthermore, over the time-course of this experiment I15HBC fails to achieve net DNA synthesis at 37°C, although it does plaque at this temperature. None of the ts or recombinant viruses assayed replicate DNA at 39.5°C as measured in this assay, although I15HBC is capable of forming minute syn^- plaques at this temperature. Replication of I15HBC DNA may be obscured if the proportion of infectious input DNA is small as a consequence of a high particle to pfu ratio. It is interesting that although few deficiencies in viral gene expression at NPT could be detected (Section 3F4) none of the viruses could replicate DNA. As Vmw175 is reportedly not directly required for DNA replication (see Section 1B3) this might suggest that Vmw175 has a critical role in controlling the expression of replication functions, and that this function differs somehow from the activation of other early genes.

6. Site-specific DNA binding by Vmw175 produced by recombinant viruses

Nuclear extracts of virally infected Flow HeLa cells were assayed for Vmw175 site-specific DNA binding activity to the IE3 cap site as described above (Section 3D) using

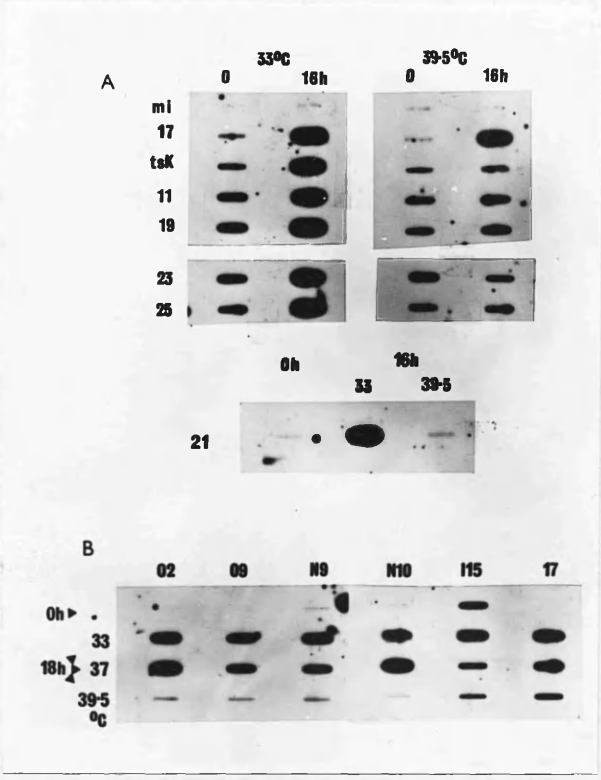


Figure 42: DNA synthesis by the recombinant and temperature sensitive viruses at the permissive and non-permissive temperatures. The ability of recombinant and ts viruses to replicate viral DNA was examined at permissive (33°C) or NPT (39.5°C). Total cellular DNA was prepared immediately post absorption (0h) or 16-18h p.a. and viral DNA quantified by dot blot analysis using primer extended pGX156 DNA as probe. (pGX156 carries the EcoRI h fragment which spans U_S). Assays are shown for HSV-1 strain 17^+ (17), tsK, ts1211 (11), ts1219 (19), ts1223 (23), ts1225 (25), ts1221 (21), 02AAA, 09CBA, N9BAA, N10DAA, and I15HBC; all infected at 5pfu per cell. All viruses, except 17^+, failed to replicate their DNA significantly at 39.5°C, although DNA replication by 17^+ itself is reduced at this temperature (panel B). All viruses replicated DNA at 33°C, although I15HBC was inefficient in this experiment. (I15HBC would also seem to have a high particle to infectious pfu ratio on the basis of this experiment, as the amount of input viral DNA at 0h pa is much greater than with the other viruses.) The 02/09 and N9/N10 recombinants synthesized DNA at 37°C, whilst I15HBC fails to accumulate a significant net gain in viral DNA at this temperature in this experiment.

the gel retardation assay. As shown in Figure 43 this assay can be used to characterize mutants temperature-sensitive for DNA binding by varying the temperature of the incubation. In this case Vmw175 present in extracts made from cells infected with tsK was able to bind to DNA at 0, 20 and 30°C, but when the incubation was carried out at 38.5°C (the NPT for viral growth) no Vmw175-containing complex "a" was formed. The phenotype of the extracts was identical whether the viral extracts were prepared at the PT or NPT. The failure of tsK-Vmw175 to bind to the IE3 cap site at NPT correlates with the failure of this virus to autoregulate IE gene 3 expression *in vivo* at the NPT.

(i) ts viruses

Nuclear extracts prepared at PT or NPT from HeLa cells infected with each of the five ts mutants ts1211, 1219, 1221, 1223 and 1225 were able to form specific Vmw175/DNA complexes when incubated at 33°C (Figure 44A). However, at 39.5°C only the ts1225 extract could efficiently form the complex "a". This complex was still formed at 40°C, although at these temperatures both ts1225 and wild-type Vmw175 bind less strongly. Furthermore, the complex formed by ts1225-Vmw175 shows identical sensitivity to increasing salt concentration as the wild-type complex, at both PT and NPT (Figure 44B). Thus although ts1225 overexpresses Vmw175 at the NPT *in vivo*, this is not correlated with an *in vitro* failure to bind to the IE3 cap site.

(ii) O and N recombinant viruses

Vmw175 in extracts prepared from cells infected with the recombinant virus O2/O9 bound to the IE3 probe as efficiently as wild-type Vmw175. However, N9/N10 encoded Vmw175 is temperature sensitive for site-specific DNA binding (Figure 45). Thus the mutant allele of IE3 present in O2/O9 behaves as predicted from experiments with its parent plasmid pI9, which contains a twelve bp insert at base 752. The temperature sensitivity of N9/N10-Vmw175 DNA binding activity was not expected from initial studies on its parent, pI39 (insert at amino acid 1239), but further analysis of the binding activity detected in nuclear extracts of cells transfected with this plasmid confirm that this too is temperature sensitive (Figure 45). At the NPT a

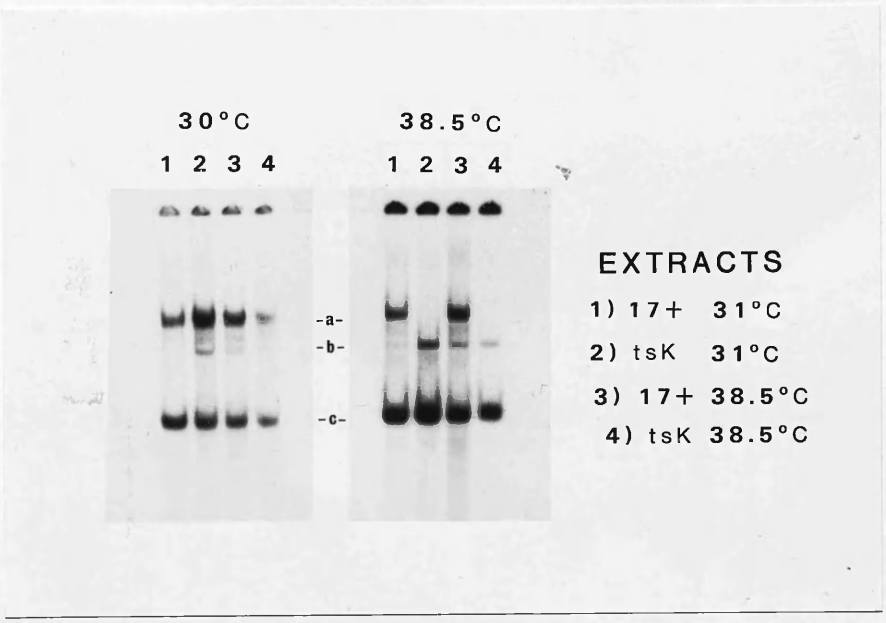


Figure 43: The HSV-1 mutant tsK produces Vmw175 with temperature sensitive DNA binding properties. Nuclear extracts of wild-type (17^+) and tsK HSV-1 infected cells were prepared 5h pa at both the permissive (31°C) and nonpermissive (38.5°C) temperatures. DNA binding to the IE3 cap site was assayed as described above (Section 3D) at 30°C and 38.5°C. Formation of the tsK-Vmw175/DNA complex was found to be ts whether the virus was grown at NPT or PT.

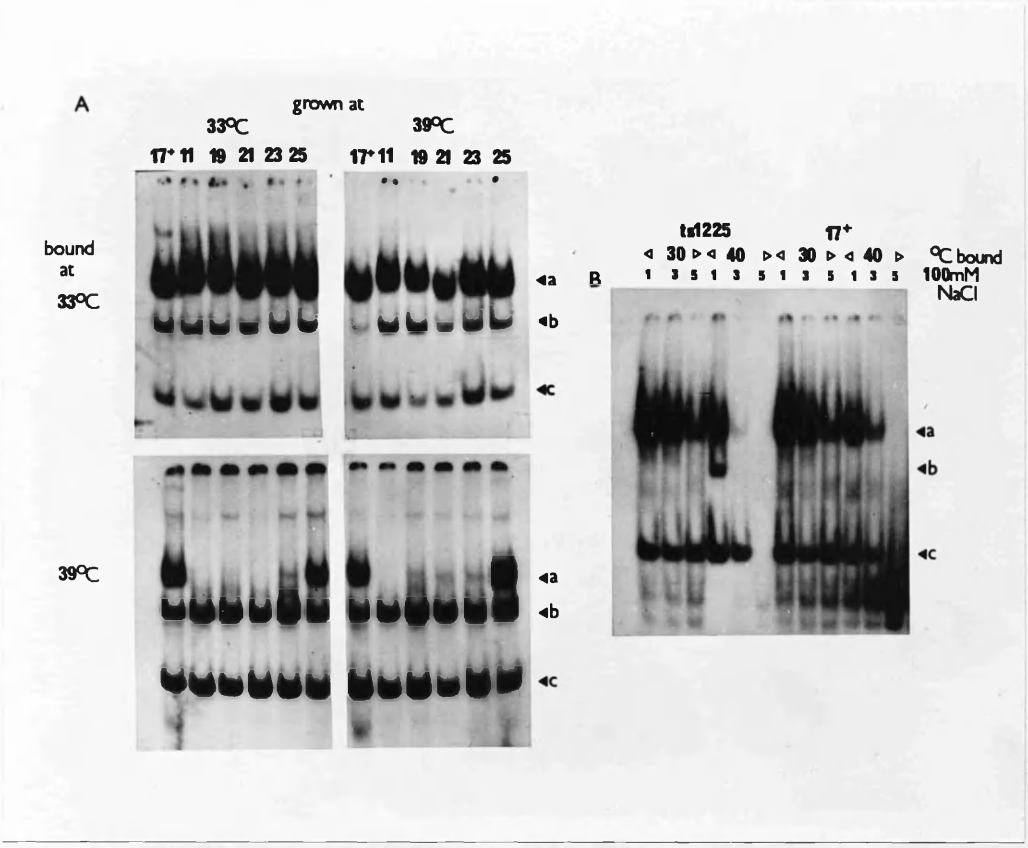


Figure 44: The site-specific DNA binding activities of Vmw175 synthesized by the ts viruses at permissive and non-permissive temperatures. (A) Total cell extracts were prepared from cells infected at PT (33°C) and NPT (39°C) with HSV-1 17^+ and the five ts viruses (ts1211, 1219, 1221, 1223 and 1225). These were assayed for DNA binding to the IE3 cap site at 33°C and 39°C. Whereas all of the ts-Vmw175 proteins were capable of forming complex "a" when assayed at 33°C, only ts1225-Vmw175 bound efficiently to DNA at 39°C (although some of the other mutants seemed to bind DNA weakly at this temperature). The Vmw175/DNA complex ("a") formed by some of the ts mutants, notably ts1211 and ts1219, was of reduced mobility on the gel, indicating structural differences between mutant and wild-type polypeptides. (B) The salt-sensitivity of ts1225-Vmw175 DNA binding was investigated at 30°C and 40°C and compared with wt(17^+)-Vmw175. Binding was carried out in the presence of 100, 300, and 500mM NaCl. The salt-sensitivity of wt and ts1225 Vmw175 was shown to be very similar at both temperatures, with the ts protein perhaps binding marginally less strongly at the elevated temperature. It is interesting to note the appearance of a novel uncharacterized complex, running below "c", when wt-Vmw175 complex "a" is dissociated at high temperature and salt concentration. This may be related to the complex "z" seen in Figure 19B, but has not been investigated further.

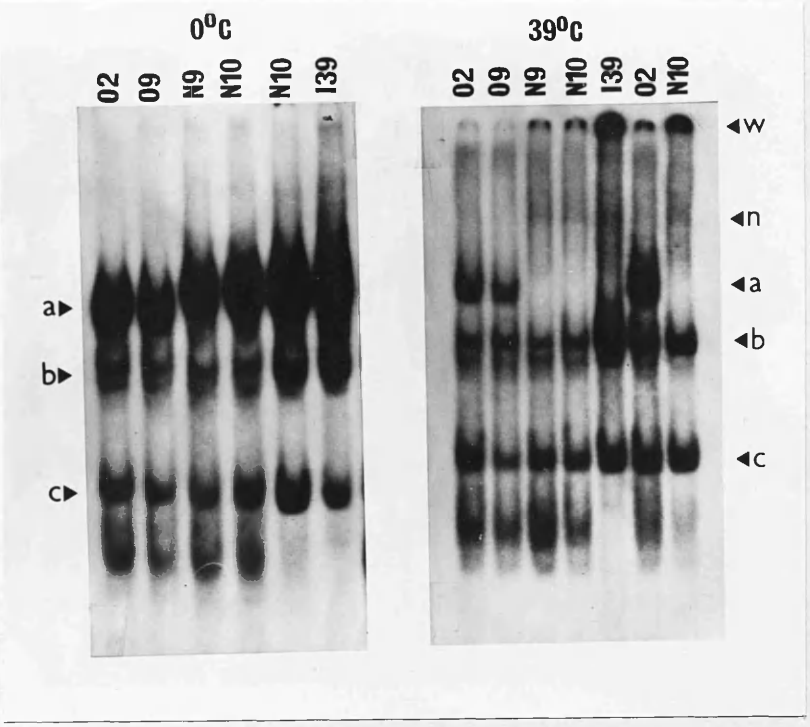


Figure 45: The site-specific DNA binding activities of Vmw175 synthesized by the recombinant viruses at permissive and non-permissive temperatures. Vmw175 in total cell extracts of Flow HeLa cells infected with the two viral Vmw175 insertion mutants (02/09 and N9/N10) bound like wt-Vmw175 to the IE3 cap site, assayed as previously described at 0°C. The Vmw175 produced in extracts of cells transfected with parent plasmid of the N9/N10 insertion mutant, pI39, also bound to DNA at 0°C but like the virally expressed protein failed to form the Vmw175-DNA complex ("a") at 39°C. At this elevated temperature there was evidence that this mutant ts protein was binding weakly to the probe to form abberant complexes of reduced mobility "n" and "w". As discussed with the abberant complexes formed by other insertion and deletion mutants of Vmw175 mapping towards the carboxy-terminus of the protein (see Figure 28) this might be caused by partial denaturaton of the protein at the carboxy-terminus, causing the complex to migrate more slowly into the gel.

slight degree of complex formation is still observed with these extracts which runs into the gel with somewhat reduced mobility ("n"), and seems to be trapped in the wells to some extent ("w"). This phenotype resembles that seen with other mutants towards the C-terminus of Vmw175 (pI29, pD9, pD11 and pD12) and it has been speculated that this may reflect a denaturing of the C-terminus region of the protein, which does not seem to be directly involved in DNA binding. As with tsK the failure of N9/N10-Vmw175 to bind DNA *in vitro* correlates with IE gene overexpression *in vivo* at the NPT; whereas O2/O9-Vmw175 does bind DNA at the NPT and does not markedly overexpress IE genes *in vivo*.

(iii) I15 recombinant virus

The recombinant virus I15HBC has previously been shown to grow poorly especially at elevated temperatures, and less ELISA detectable Vmw175 and Vmw175/DNA binding activity could be prepared from cells infected with I15HBC than with other viruses (results not shown). However, I15HBC-Vmw175 did seem to bind the IE3 probe as efficiently at 39°C as at 0°C, (Figure 48). The complex formed was identical to that formed by Vmw175 expressed from its parent plasmid pD1 which is deleted from bases 483 to 686 of the IE3 ORF (amino acids 162-229) (Figure 46, see also Figure 20). Treatment of the I15-Vmw175 DNA complex with increasing amounts of proteinase K (as described in Section 3D1.4) produced a novel retarded band "p" of identical mobility to that formed from wild-type Vmw175 (Figure 47). Thus if this complex is composed of the minimal DNA binding domain it is unlikely that amino acids 161-227 are part of this domain, or complex "p" would be of reduced size when formed from I15-Vmw175. The proteinase K treated complexes obtained with pD1-expressed Vmw175 were identical to the I15-expressed complex.

(iv) rescued viruses

Nuclear extracts were prepared from HeLa cells infected with viruses rescued from mutants (described in Section 3F3 above) and assayed for Vmw175/DNA binding activity (Figure 48). Each of the rescued viruses behaved like wild-type, confirming that the DNA binding phenotype had been due to the rescued lesion, not a second site mutation.

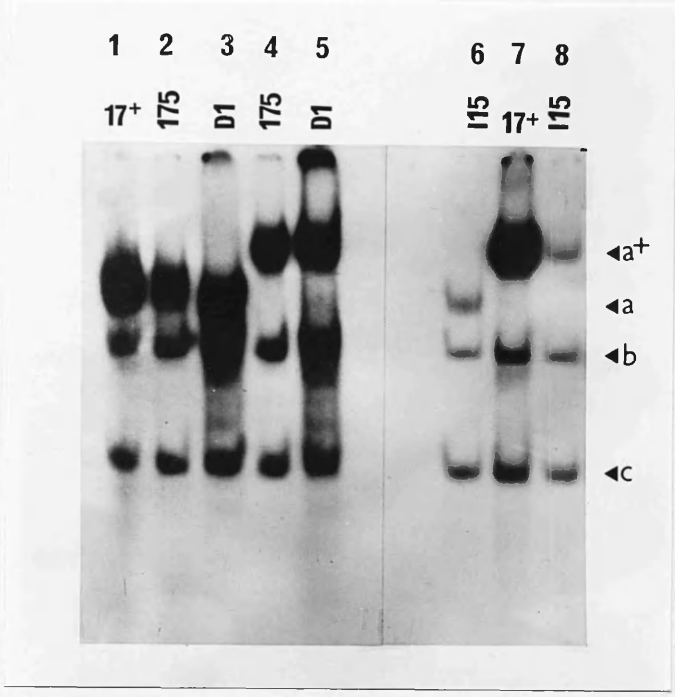


Figure 46: Vmw175 specified by the recombinant virus I15HBC binds to DNA to form a complex identical to that specified by its parent plasmid pD1. DNA binding of mutant Vmw175 expressed by the recombinant virus I15HBC was assayed at 20°C and the Vmw175-DNA complexes produced compared to those produced by HSV-1 strain 17^+ infected cells (17^+) and p175 and pD1 transfected cells. Reactions 4, 5, 7 & 8 were further incubated with 1ul of (1/50) 58S antibody before electrophoresis. Like the complex produced by the parental plasmid-borne mutation, the I15-Vmw175/DNA complex was marginally smaller than the wt complex "a" (see also Figures 20 and 48).

Figure 47: An identical proteolytic fragment of Vmw175 binds to DNA when synthesized by wild-type virus, the recombinant I15HBC or the plasmid pD1. The production upon protease K (PK) digestion of the novel protein-DNA complex "p" was assayed as described in Figure 23. Extracts were made from cells infected with HSV-1 17^+, I15HBC, or transfected with pD1.

Lane	Extract	PK	Antibody 2180V
A1	17^+	0ng	0
A2	17^+	0ng	3ul
A3	17^+	20ng	0
A4	17^+	20ng	3ul
A5	17^+	100ng	0
A6	17^+	100ng	3ul
A7	I15	0ng	0
A8	I15	0ng	3ul
A9	I15	20ng	0
A10	I15	20ng	3ul
A11	I15	100ng	0
A12	I15	100ng	3ul

Lane	Extract	PK
B1	17^+	0ng
B2	17^+	1ng
B3	17^+	10ng
B4	I15	0ng
B5	I15	1ng
B6	I15	10ng
B7	p175	0ng
B8	p175	1ng
B9	p175	10ng

The results show that the proteolytically produced complex "p" is identical whether specified by wt or I15 virus, or by the parent plasmid pD1. Furthermore, in this experiment I15-directed complex "p" may be formed somewhat more efficiently than the native I15-Vmw175 complex "a". Because the three independent "p" complexes are of apparently identical size, the proteolytic fragment of Vmw175 in "p" probably does not include the region of the D1/I15 deletion (amino acids 162-229).

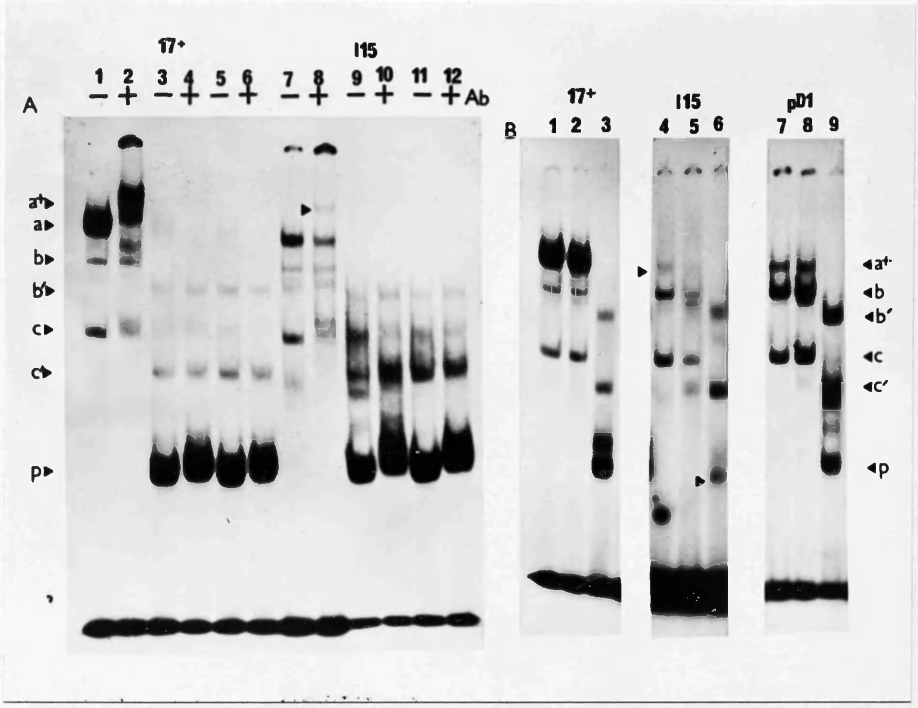
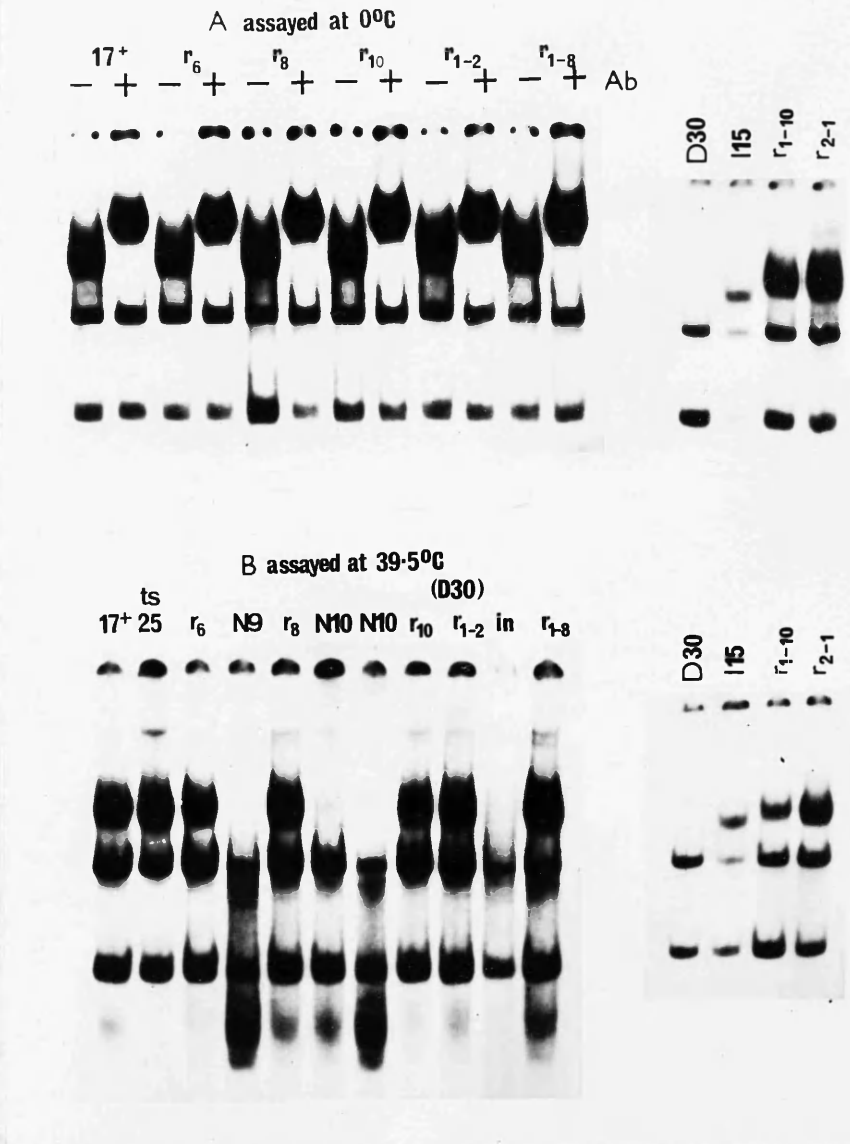

A
17+
I15
1 2 3 4 5 6 7 8 9 10 11 12 Ab
a+
a
b
b'
c
c'
p
B
17+
I15
pD1
1 2 3 4 5 6 7 8 9
a+
b
b'
c
c'
p

Figure 48: Vmw175 synthesized by viruses marker rescued from the recombinant and ts viruses binds to DNA at both permissive and non-permissive temperatures. Each of the rescued viruses described in Table 15 were assayed for Vmw175-DNA binding activity at 0°C (A) and 39.5°C (B). Vmw175 in infected cell extracts of all of the rescued viruses bound to the IE3 probe to form complex "a" (-) which was shifted to complex "a^+" upon further incubation with 58S antibody prior to electrophoresis (+). At 39.5°C each of the mutant polypeptides behaved as previously described; no Vmw175/DNA binding activity was detectable in extracts of cells infected with the N9, N10, in1411 and D30EBA recombinants. However, rescued isolates of these viruses (in adjacent tracks) were able to direct production of the Vmw175/DNA complex. Also note that I15HBC-Vmw175 could bind to DNA at the elevated temperature, and that the ("a") complex formed by this protein is marginally smaller than that formed by both its rescued clones (r1-10 and r2-1).

Mutant and rescued viruses are as follows: ts1225/r6; N9BAA/r8; N10DAA/r10; D30EBA/r1-2; in1411/r1-8; I15HBC/r1-10 (XhoIC rescued)/r2-1 (rescued with a fragment internal to the IE3 gene, -126 to +2041).


A assayed at 0°C
17+
r6
r8
r10
r1-2
r1-8
Ab
D30
I15
r1-10
r2-1
B assayed at 39·5°C
ts
17+ 25
r6
N9
r8
N10
N10
(D30)
r10
r1-2
in
r1-8
D30
I15
r1-10
r2-1

CHAPTER 4: FURTHER STRUCTURAL ANALYSIS OF THE Vmw175 RELATED POLYPEPTIDES

1. Features of the genes encoding the Vmw175-related polypeptides.

The sequence of HSV-1 IE gene 3 and its counterparts from VZV and PrV has been determined and the amino acid sequence of the encoded proteins predicted (McGeoch et al., 1986; Davison and Scott, 1986; C.Vlcek, M.Schwyzer and V.Paces, per.comm.). Each of the three DNA coding sequences is markedly G+C rich (Table 16). The HSV and PrV DNA sequences are remarkably G+C rich throughout the entire genome, but particularly high in this gene. The G+C content of the VZV 140K ORF is less extreme, but greatly exceeds that of the VZV genome as a whole.

The amino acid composition of the three proteins is similarly biased towards amino acids encoded by G+C rich codons, in particular the amino acids glycine, alanine, arginine and proline (GARP) are extremely abundant in all three proteins (Table 17). It would appear that HSV and PrV at least have evolved to maximize G+C content to the greatest amount possible in this gene without interfering with protein function, and that evolutionary forces have acted to maximize the high G+C content by favouring amino acids with G+C rich codons and by favouring synonymous codons with G or C in the third position. Consequently amino acids with A+T rich codons appear to be under-represented (for example leu, asn, ile and met) (McGeoch et al., 1986). It has also been suggested that regions of particularly high GARP content, including large parts of regions 1 and 3, may be relatively unimportant functionally, this would appear to be borne out by the results in this thesis.

2. Homologies between Vmw175 and other herpesvirus transactivating IE proteins.

As discussed in Section 1D1.5 the three proteins Vmw175, VZV 140K and PrV IE protein are highly homologous in three regions A, B and C (Figure 5). The proteins have been divided into five potential structural regions (1-5) on the

Table 16: Base composition of the IE genes.

	IE gene ORF	Viral chromosome
VZV	64.1% G+C	46.0% G+C
HSV	81.4% G+C	68.3% G+C
PrV	79.8% G+C	73% G+C

The base composition of the three sequenced IE genes is given, compared to that of the total virus chromosome. (Davison and Scott, 1986; McGeoch et al., 1986, 1988; Ben-Porat and Kaplan, 1962; C.Vlcek, M.Schwyzer and V.Paces, per.comm.).

Table 17: Amino acid composition of the IE protein.

	A ala	+	R arg	+	P pro	+	G gly	=		S ser
HSV	20.9		10.7		12.4		10.9	=	54.9	5.9
VZV	10.9		9.7		11.3		9.2	=	41.1	9.2
PrV	17.4		11.1		12.4		10.7	=	51.6	9.2

The percentage occurence of common residues in the predicted amino acid sequences of the three IE proteins (HSV-1 Vmw175, PrV IE protein and VZV 140K ORF) is given. (From the sequence data of McGeoch et al., 1986; Davison and Scott, 1986; C.Vlcek, M.Schwyzer and V.Paces, per.comm.).

basis of these blocks of homology (Figure 49; Tables 18 and 19). It has been predicted that these conserved regions might correspond to functionally important regions of the proteins.

3. Interesting structural features of the protein sequences.

3.1 Homology region A.

This highly conserved region is composed of a serine rich tract followed by acidic residues (asp and glu) (Figure 50). It has been proposed that these serines are the major phosphorylation site for Vmw175 (DeLuca and Schaffer, 1988). Phosphorylation could play an important role in regulating protein activity and differentially phosphorylated forms of Vmw175 may have different biochemical properties (Michael et al., 1988). When phosphorylated this region would be highly acidic, and could perhaps function as an acidic transcription activating region (Section 1E3.3). However, the results presented in this thesis show that this region is not absolutely required for either transactivation or repression activity in transfection assays (Section 3C3) and that a virus deleted for this region of the protein is viable on non-complementing cell-lines (Section 3F).

3.2 The carboxyl terminus.

The non-conserved region 5 varies in size between the three proteins, (Figure 49, Table 18). However, some similar structures can be found within this region. Each protein includes an acidic string of 6-9 residues near to its carboxy-terminus (Figure 51a). The VZV acidic region also includes a short polyserine tract and may in fact be related to homology region A (see below, 3.4). An alternative relationship between the carboxyl termini of Vmw175 and VZV 140K has been suggested, with the terminal residues of Vmw175 being conserved 80 residues upstream from the VZV 140K terminus (Figure 51b, McGeoch et al., 1986).

3.3 Potential nuclear localization signals.

Sequences necessary for the nuclear localization of Vmw175 have been mapped to within amino acids 682-774 (this thesis, Section 3B). Within this sequence is a short basic

Amino Acid Sequence Comparison between the major immediate early proteins of Pseudorabies, Herpes simplex and Varicella Zoster Virus

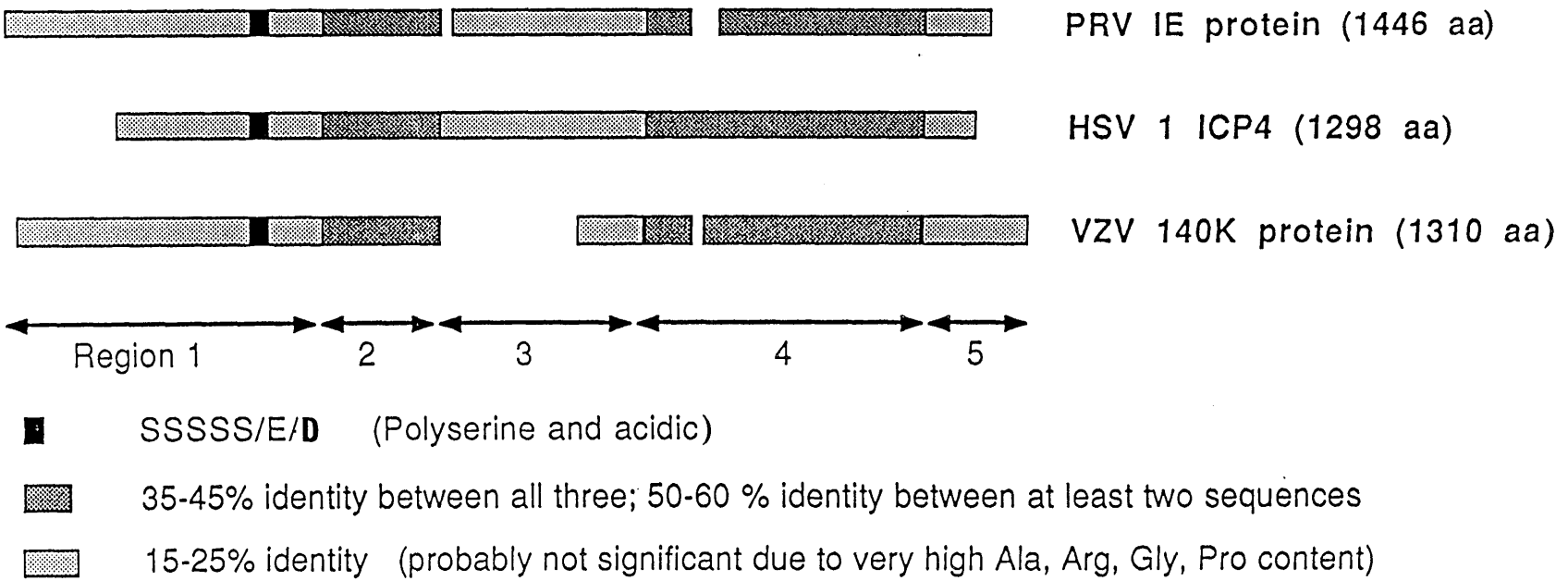


Figure 49: Amino acid sequence comparison between the major immediate early proteins of pseudorabies, herpes simplex and varicella zoster virus. (After Vlcek, Schwyzer and Paces, per.comm). The three protein sequences are aligned to show the two major and one minor conserved regions (regions 2 and 4 and homology region A, the polyserine/acidic tract).

Table 18: The five regions of the conserved IE proteins.

REGION	HSV-1 Vmw175	PrV IE protein	VZV 140K ORF
1	1-314 (314)	1-470 (470)	1-467 (467)
2	315-484 (170)	471-659 (189)	468-641 (173)
3	485-796 (312)	660-957 (298)	642-734 (92)
4	797-1224 (428)	958-1350 (393)	735-1148 (414)
5	1225-1298 (74)	1351-1446 (96)	1149-1310 (162)

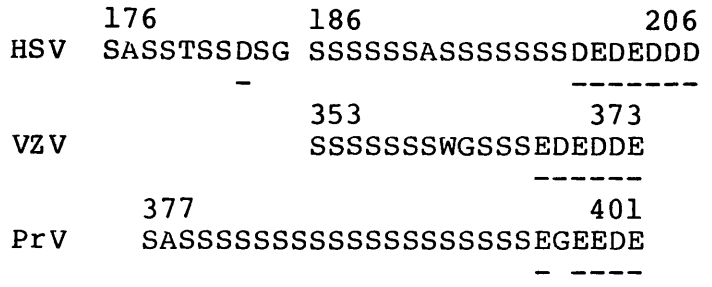
The five regions of the three homologous alphaherpesvirus IE proteins are shown, numbered in amino acids, as defined by C.Vlcek, M.Schwyzer and V.Paces (per.comm.). The sizes of each region are given in parenthesis.

Table 19: Conservation of regions 2 and 4 between the 3 IE proteins.

	Region 2	Region 4
Identity between PrV/HSV/VZV	36%	45%
Additional identity between:	Region 2	Region 4
PrV/VZV	17%	12%
PrV/HSV	15%	10%
HSV/VZV	10%	11%

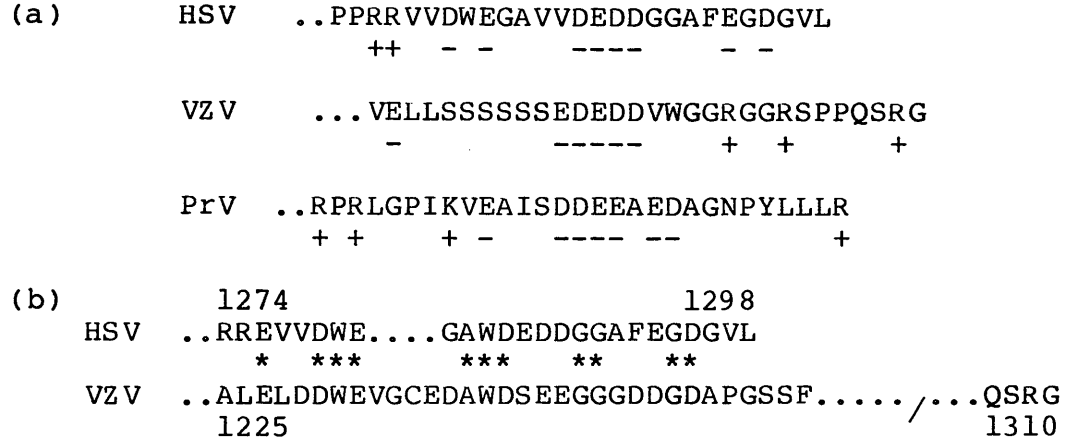
(Data of C.Vlcek, M.Schwyzer and V.Paces, per.comm.). The proportion of regions 2 and 4 conserved as identical amino acids between each of the three IE proteins (HSV-1 Vmw175, PrV IE protein and VZV 140K ORF) is shown. Regions 2 and 4 are 35-45% identical between all three proteins, whilst regions 1, 3 and 5 are 15-25% identical, which mainly reflects the high ala+arg+gly+pro content of the proteins as a whole. (The amino acid identity between PrV and VZV in region 2 therefore is 36 + 17 = 53%).

Figure 50: The structure of homology region A.



The amino acid sequences of the serine rich homology region A is given for the three IE proteins HSV Vmw175, VZV 140K and PrV IE. The deletion in the HSV IE3 ORF in plasmid pD1 and the recombinant virus I15HBC spans amino acids 162-229. The spontaneous deletion described by Schroder et al., (1985) spans amino acids 209-236. The PrV IE protein also has a highly serine rich stretches between amino acids 830 and 953. The potential charge on each amino acid (+/-) is shown below each residue.

Figure 51: A conserved region at the carboxy-terminus of the IE proteins?



(a) The predicted amino acid sequence is shown for the carboxyl-terminus of each of the three IE proteins HSV Vmw175, VZV 140K and PrV IE. Each protein has a short run of acidic (D or E) residues close to the carboxyl terminus. In the VZV protein this structure closely resembles that of homology region A, with a serine string preceding the acidic run. The potential charge on each amino acid (+/-) is shown below each residue. (b) An alternative relationship between the carboxy-termini of the HSV and VZV proteins was suggested by McGeoch et al., (1986). The sequence at the terminus of Vmw175 was found to be similar to a region 80 residues upstream from the 140K terminus. These sequences are aligned and identities asterixed.

string of amino acids RKRK which is related to nuclear localization signals identified in other proteins (Kalderon et al., 1984) and is conserved in VZV 140K (Figure 52). This sequence is not identically conserved in the PrV sequence, but related basic strings are found in the equivalent area of region 3, which could be involved in nuclear localization.

3.4 Repeated amino acid strings.

Related amino acid strings are found within each of the three Vmw175-related proteins. It is not clear whether these tru ly represent the result of ancestral duplications within the coding sequence or are merely a consequence of the high GARP content of the proteins. These repeats were analyzed in detail using the COMPARE program to search for repeated amino acid strings within each protein, scoring for identical amino acids, with varying stringencies. Far fewer apparent repeats were detected in the VZV protein sequence at a given stringency, probably reflecting the lower GARP content of this protein.

Some of the more interesting repeated strings are shown in Figure 53, aligned by the BESTFIT program. Elements (i) and (iii) may be examples of sequences picked out as homologous on the basis purely of their high GARP content. Element (ii) identified in the PrV protein is more interesting as it identifies a second serine rich area of this protein between residues 830 and 875. The relationship between this sequence and homology region A (370-400) is probably simply a reflection of the high serine content of both proteins. The VZV homology region A is also picked out as a possible repeated element by this analysis (element iv) and appears to be repeated at the carboxy-terminus of this protein. Vmw175 does not posses either of these second serine rich regions, and has a much lower serine content than the other two proteins (Table 17).

3.5 Acidic regions.

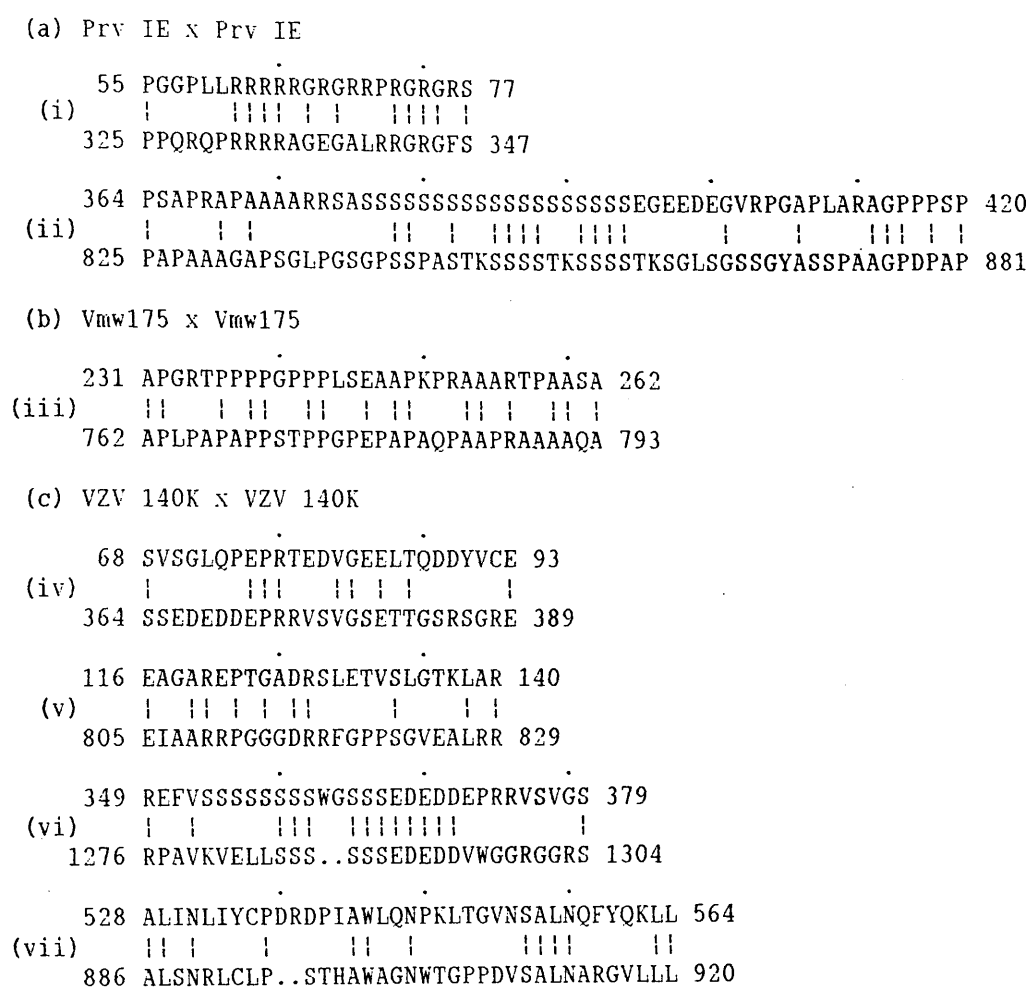
Because of the identification of protein domains rich in acidic amino acid side chains as transcriptional activating regions (Section 1E3.3) it was interesting to examine each

Figure 52: The potential nuclear localization signals of the IE proteins.

	727	
HSV	RKRKS	
	682	
VZV	RKRKS	
	883	927
PrV	RRKKKRR	RRKRR

The amino acid sequences of potential nuclear localization signals in region 3 of the IE proteins are shown. The HSV sequence is within the boundaries of a region identified as essential for nuclear localization (this thesis; DeLuca and Schaffer, 1988) and is closely conserved in VZV. Strings of basic residues in the similar region of the PrV protein are also shown.

Figure 53: Repeated amino acid strings in the Vmw175-related proteins.



Repeated amino acid strings were identified within the three IE proteins by analyzing their sequences using the COMPARE program, scoring for identical amino acids. (a, PrV IE protein; b, HSV-1 Vmw175; c, VZV 140K protein compared to themselves). A window size of 30 was used, with increasing stringency for matches between 13 and 17. Identified repeated strings were aligned by the BESTFIT program, as shown above. Most of the repeat elements identified were of questionable significance and probably only reflect the high GASP composition of these proteins.

of the three Vmw175-related proteins for unusually charged regions. Figures 54-56 show the amino acid sequences of the three proteins with the potential charge on their side chains indicated. Aspartic and glutamic acid are considered as acidic; whilst lysine, arginine and to a lesser extent histidine are considered basic.

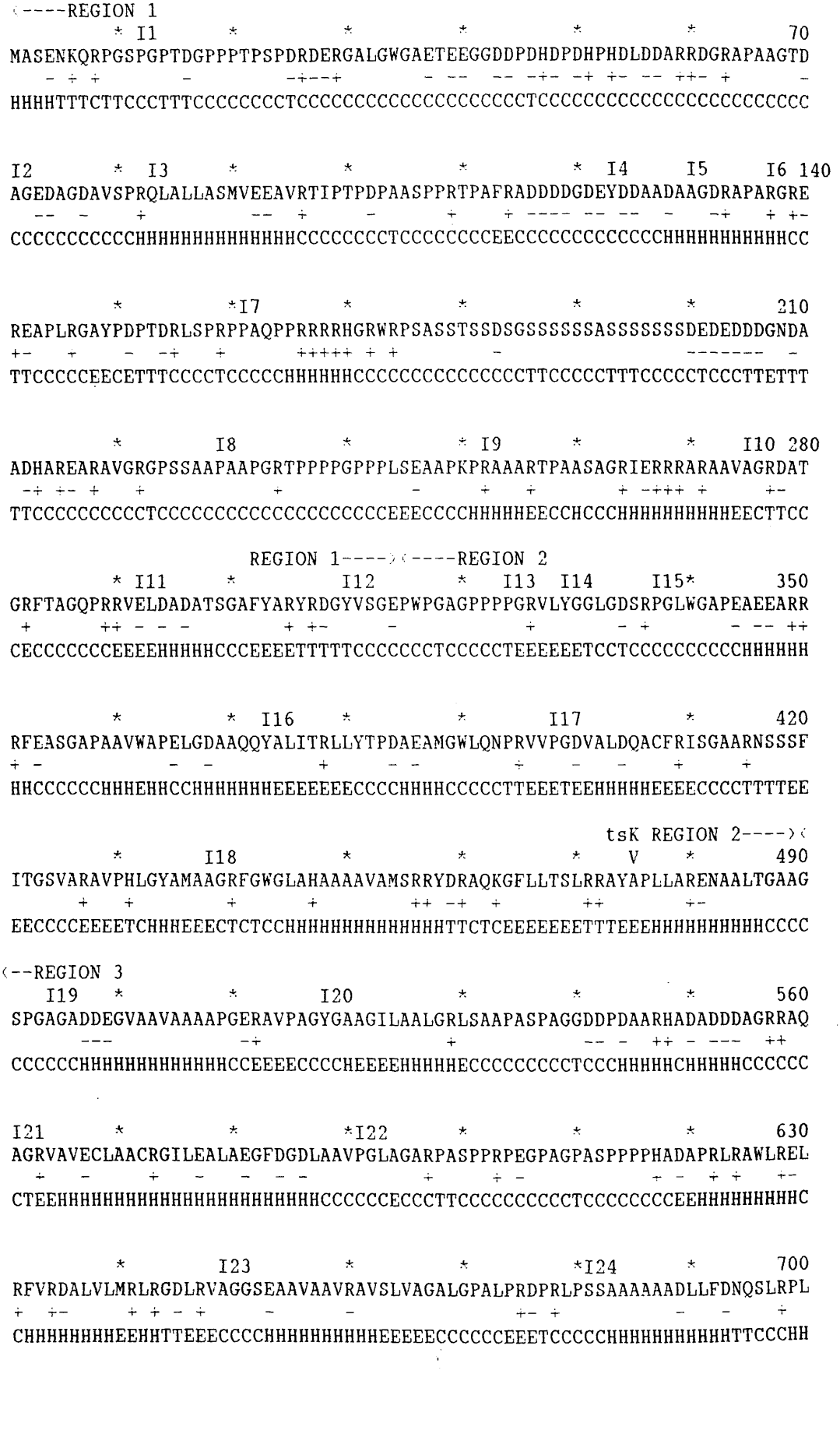
The regions of the protein identified in Chapter 3 as the most important for activation function (regions 2 and 4) do not contain any acidic stretches in any of the proteins. Conserved acidic regions are found in region A (Figure 50) and towards the carboxyl terminus (Figure 51a). Both of these regions have been shown not to be essential for activation in transfection assays, and a virus deleted for homology region A was viable in non-complementing cells. A region of the VZV 140K protein (amino acids 1230-1290) is particularly acidic, with a net charge of -12, but is not conserved in Vmw175. Somewhat acidic stretches were also found towards the amino terminal of the three proteins (HSV: 115-130; VZV: 79-99; PrV: 1-40 and 95-120). Again these regions are not conserved between the proteins.

The lack of obvious acidic stretches in regions 2 and 4 of the proteins may indicate that activation of transcription by these proteins does not occur by this mechanism. Alternatively acidic regions in other areas of the protein may not have been recognized as important if insertion of four amino acids into these regions fails to disrupt activity, or if there are multiple acidic regions capable of activation. It would be interesting therefore to construct mutants of Vmw175 deleted for all of the acidic strings and to determine whether these mutants were still able to activate transcription.

It is also worth noting that there are no obvious glutamine rich motifs in Vmw175, although glutamine rich domains have been proposed as a novel type of activating domain in Sp1 and other proteins (Courey and Tjian, 1988). The amino terminal 300 residues of VZV 140K are somewhat glutamine rich, and a glutamine pentapeptide is present in the PrV IE protein (Figures 54-56).

Figure 54

PREDICTED PRIMARY AND SECONDARY STRUCTURE OF HSV-1 Vmw175

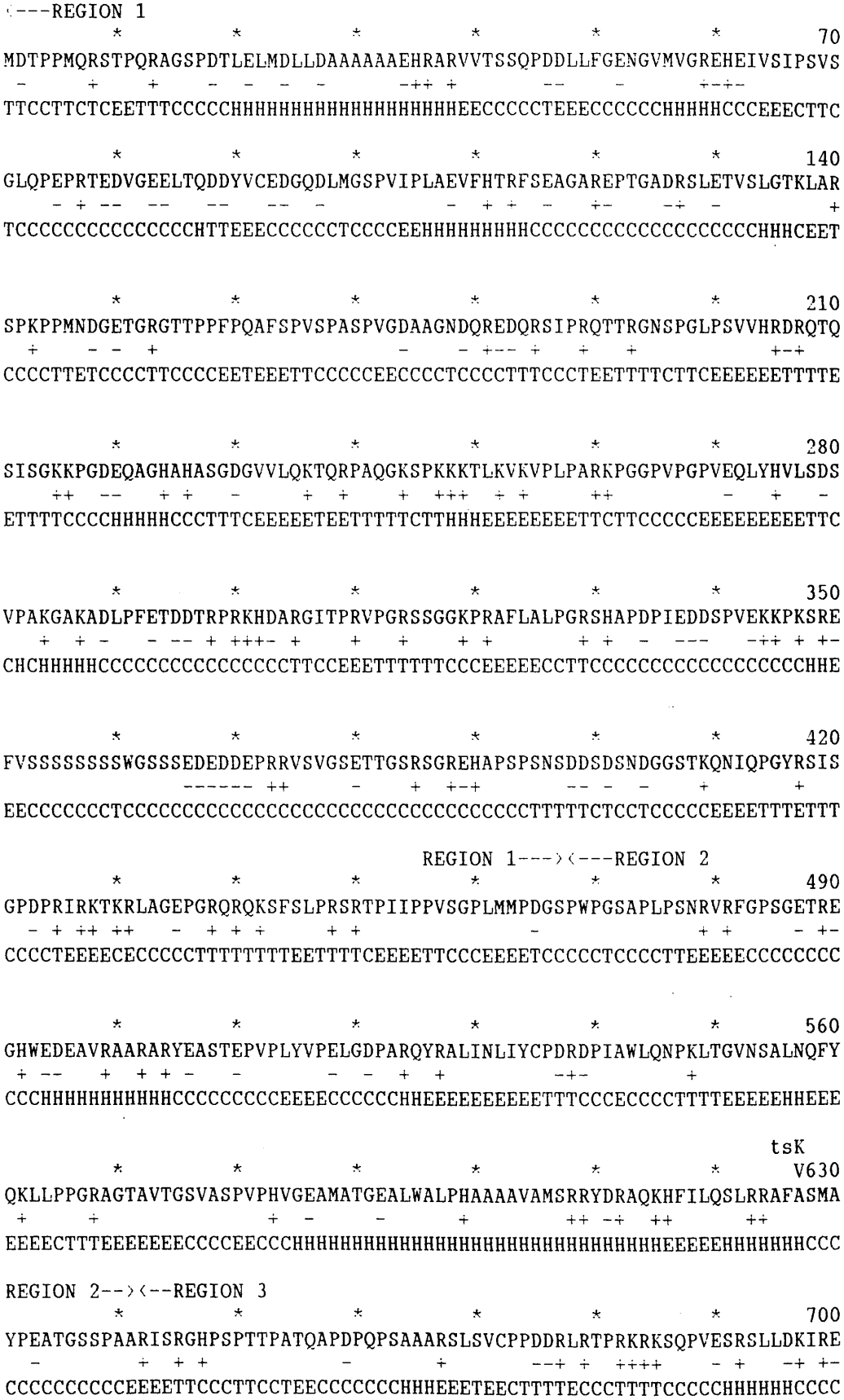


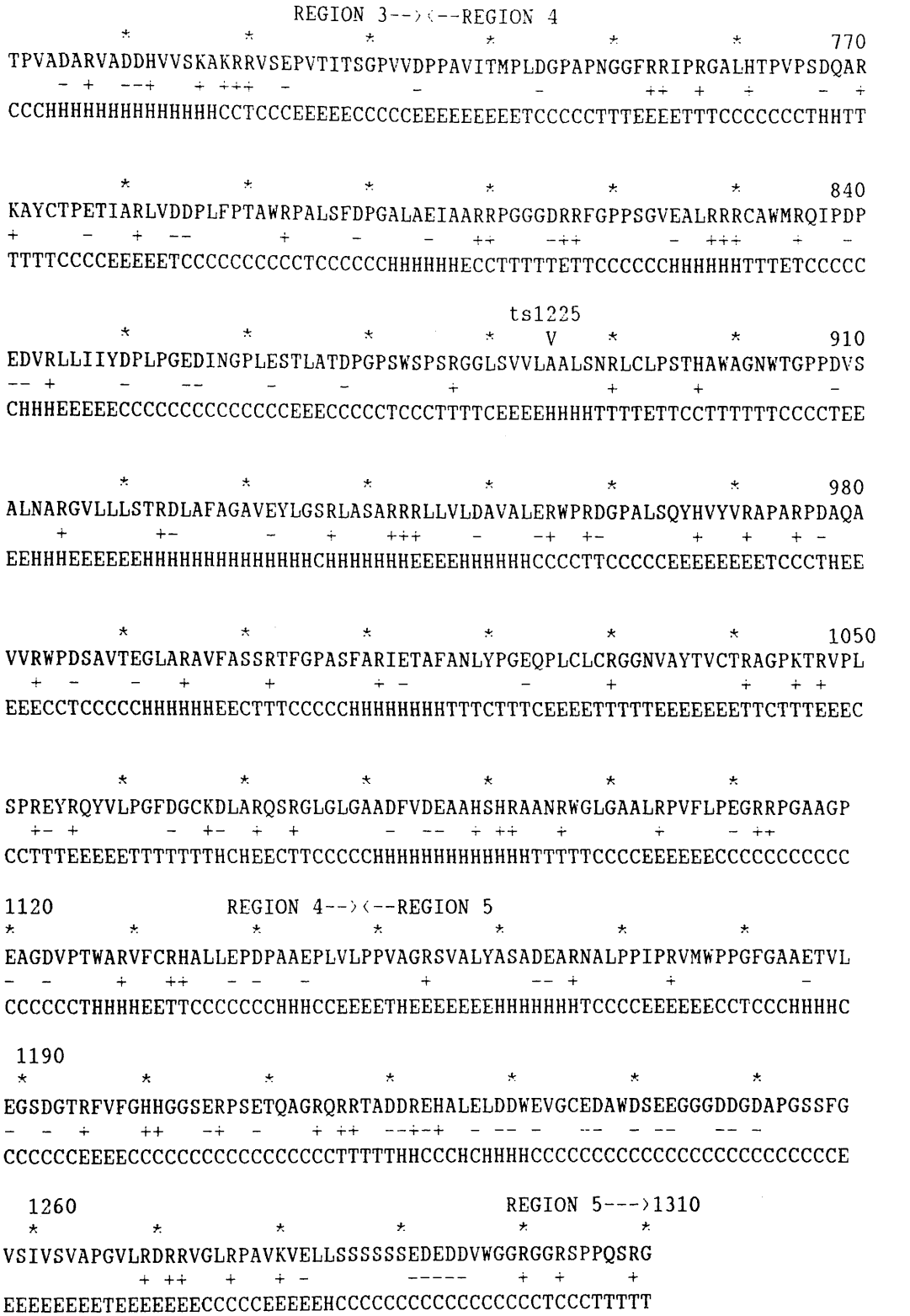
```
         *         *         *         *         *         *        770
LAAAASAPDAADALAAAAASAAPREGRKRKSPGPARPPGGGGPRPPKTKKSGADAPGSDARAPLPAPAPP
        -  -              +- ++++     +          + ++   -    - +
HHHHHHHHHHHHHHHHHHHHHHHHHTTTTTCCCCCEETTTCCCCCTTCTECCCCCCCCCCCECCCCCCCT

              REGION 3---->(----REGION 4
   I25   *      I26*         *  I27    *         *         *        840
STPPGPEPAPAQPAAPRAAAAQARPRPVAVSRRPAEGPDPLGGWRRQPPGPSHTAAPAAAALEAYCSPRA
      -         +      + +     ++  -  -     ++       +          -     +
TCCCCCCCCCCCHHHHHHHHHHCECEEEEEECCCCCCCCTTTTCCCCTCTTCEEHHHHHHHHHTTCHHH

  I28     *         *         *         *         *         *        910
VAELTDHPLFPVPWRPALMFDPRALASIAARCAGPAPAAQAACGGGDDDDNPHPHGAAGGRLFGPLRASG
  -  -+       +     - +       +                 ----  + +     +      +
HHHHTCCCCCCCCCCCTEEEHHHHHHHHEEETTCCCHHHHEETTTTTTTCCCCCCCCCTTEEEEECCTCC

                                                      ts1225
         *         *   I29   *  I30    *         *     V   *        980
PLRRMAAWMRQIPDPEDVRVVVLYSPLPGEDLAGGGASGGPPEWSAERGGLSCLLAALANRLCGPDTAAW
  ++     +   - -- +           --              -   -+           +    -
CCEHHHHHETCCCCCCEEEEEEECCCCCCEECCCCCCCCCCCCCCCCTTTCCEEHHHHHHTTTCTTEECC

         *         *         *        *I31      *         *       1050
AGNWTGAPDVSALGAQGVLLLSTRDLAFAGAVEFLGLLASAGDRRLIVVNTVRACDWPADGPAVSRQHAY
        -               +-       -          -++       +  -   -     + +
CCTTCCCCTEEEHHHHEEEEEEHHHHHHHHHHHHHHHHHHHHHHHHEEEEEEEETTTTCCTCCCCCHHHHTE

         *     I32 *         *         *       I33         *       1120
LACELLPAVQCAVRWPAARDLRRTVLASGRVFGPGVFARVEAAHARLYPDAPPLRLCRGGNVRYRVRTRF
  -        +    +- ++       +         + -  + +   -    +  +    + + + +
EHHHHHHHHHHEEEEEHTHHTCEEEEEEEETEECCCCEHHHHHHHHTCCTTCCTEEETTTTTEEEEEEETT

         *  I34   I35       *         *         *         *       1190
GPDTPVPMSPREYRRAVLPALDGRAAASGTTDAMAPGAPDFCEEEAHSHAACARWGLGAPLRPVYVALGR
  -       +- ++      - +       -         -  --- + +    +         +      +
CTTCCCCCCCCCCEEEEEECHHHHHCCCCCCEEECCCCCCEEEEECCCTCCEEEHHHHHHHHHHHHHCCC

                     REGION 4---->(----REGION 5
    I36  *         *         *         *I37  I38I39       *       1260
EAVRAGPARWRGPRRDFCARALLEPDDDAPPLVLRGDDDGPGALPPAPPGIRWASATGRSGTVLAAAGAV
-  +    + +  ++-   +   - ---      + ---          +      +
CCCCEEECCCHHHHHHHHHHHHHTTTTCCCCEEEEEEEHHHHCCCCCCTTCCTTTTTEECCCCCCTTCCC

         *         *         *       1298
EVLGAEAGLATPPRREVVDWEGAWDEDDGGAFEGDGVL
-    -       ++-  - -   ----    - -
CCEEETTTCCCCCCCCCCTTCCCCCCCCCCCCCCCCC
```

The predicted amino acid sequence (1-1298) of the HSV-1 protein Vmw175 is shown in the single letter amino acid code, and divided into the five structural regions predicted on the basis of homology to the VZV 140K ORF (McGeoch et al.,1986).
Secondary structure was predicted by the GARNIER program using the method of Garnier et al.,(1978) and is shown below the sequence,(C= random coil, E= beta-pleated sheet, H= alpha-helix, T= turn). Acidic and basic residues are highlighted - and + beneath the sequence. The positions of the amino acid substitutions in two temperature sensitive mutants tsK and ts1225 are shown at residues 475 (A to V) and 966 (A to T) respectively (Davison et al.,1984; R.D. Everett provisional data, per.comm.). The position of the 39 insertion mutants described in this thesis is marked above the sequence.

Figure 55

PREDICTED PRIMARY AND SECONDARY STRUCTURE OF THE VZV 140K PROTEIN





The predicted amino acid sequence (1-1310) of the VZV140K ORF is shown in the single letter amino acid code (Davison and Scott, 1986). Secondary structure was predicted by the GARNIER program using the method of Garnier et al. (1978) and is shown below the sequence, (C= random coil, E= beta-pleated sheet, H= alpha-helix, T= turn). Acidic and basic residues are highlighted - and + respectively. The equivalent positions of the HSV-1 Vmw175 ts mutations tsK and ts1225 are shown at amino acids 627 and 885 respectively. The five regions of the protein; based on homology with HSV-1 Vmw175, are shown (McGeoch et al., 1986).

Figure 56

PREDICTED SEQUENCE OF THE PRV IE PROTEIN.

```
     <---REGION 1
   1 MADDLFDFIE TEGNFSQLLA AAAAAEEEGI ASGPDGGSQG SRRRGSSGED
       --  -  -  -   *          ---    *  -  *   *+++ ** --

  51 LLFGPGGPLL RRRRRGRGRR PRGRGRSHEA APSSSAQQQQ QPRRGSGEIV
                +++++ + ++  + + +*+-    ***        ++ * -

 101 VLDDEDEEED EPGSPAAGSP GRALHQGSEH GHLVLGPRSR AGSGPRPPTP
       -------- -  *    *   +  +  *-+  +     +*+   *  +

 151 AALAAAEAGA PGGPGRSSPS AASPASSSGS PGPSAAPRRW SPARGDPVGE
           -         +** *   *  *** *    *   ++  *  + -   -

 201 PGPAARPRTP APPAQPAAVA AAPARRGPAS PASPAAGPVS APGGGGAPSA
         + +                    ++   *   *       *          *

 251 GGDRGRHHHQ HREPLLDEPA AARRLDPRPL GARSPVSSNP NSNSNSTTTV
       -+ ++++  ++-   --     ++ - +     +*  **    * *

 301 AVETVARGPE KDEDGLGLAG DGGAPPQRQP RRRRAGEGAL RRGRGFSSSS
       -   +  - +---        -       +   ++++  -    ++ +  ****

 351 SSGSDSDLSP ARSPSAPRAP AAAARRSASS SSSSSSSSSS SSSSSSSEGE
     ** *-*- *   +* *  +       ++* ** **********  *******- -

 401 EDEGVRPGAP LARAGPPPSP PAPAAAPRPS ASSASSSAAA SPAPAPEPAR
     ---  +       +     *          + *  ** ***    *     -  +
                REGION 1--> <--REGION 2
 451 PPRRKRRSTN NHLSLMADGP PPTDGPLLTP LGEPWPGSDP PADGRVRYGG
       +++++*    + *   -      -          -    *-    - + +

 501 AGDSREGLWD EDDVRQAAAR YRAAAGPVPV FIPEMGDSRK QHEALVRLIY
       -* -   - --- +    +  +              -  -*++  +-   +

 551 SGAAGEAMSW LQNPRMQAPD QRFNQFCQRR VHAPHGHGSF ITGSVTPPLP
     *    -  *      +    -  +       ++  +  + + *     *
                                                    tsK
                                                      V
 601 HIGDAMAAQD PLWALPHAVS AVAMSRRYDR TQKTFILQSL RRAYADMAYP
     +  -      -        +  *      *++ -+   +     *  ++   -

   REGION 2-->&lt;--REGION 3
 651 GRAADPRAGE ATVEALCARV RAAFAAAQPG RVPRELADAC VLACRGVLER
      +  - +  -     -    +  +           +  +-  -       +   -+

 701 LLPCPLRLPA PARAPAALGP ACLEEVTAAL LALRDAIPGA GPAERRQAAD
           +      +           --        +-          ++   -

 751 SVALVARTVA PLVRYSVDGA RAREAAWTYA AALFAPANVA AARLAEAAAR
     *     +       + * -      -                    +  -   +

 801 PGPAEPAPGL PPLWPEQPGL VVPAPAPAAA GAPSGLPGSG PSSPASTKSS
         -          -                  *    *   **  * +**

 851 SSTKSSSSTK SGLSGSSGYA SSPAAGPDPA PERRKKKRRA PGARRPGDGE
     ** +**** + *  * **    **     -   -+++++++     ++  - -

 901 EDEGLSGAAL RGDGHGHRDD EEDRGPRRKR RSLGLGPAPD PAPALLSSSS
     ---  *     + - + ++-- ---+  ++++ +*       -        ****
```

```
  REGION 3-->&lt;--REGION 4
951   SSSEDDRLRR PLGPMPEHPA PDGGFRRVPA GETHTPRPSE AALAAYCPPE
      ***---+ ++       -+    -   ++     - + + -           -

1001  VARALVDQEV FPELWRPALT FDPAALAHIA ARRGAPLRRR AAWMRQIADP
        +   - -   - +      -     +    ++    +++     +   -
                                                 ts1225
                                                    V
1051  EDVRVVVLYD PLPHEELCAE PAEGAPRPAW DPRRGGLSAL LAAFAHRLCT
      -- +     -    +--   -   -   +      ++   *        ++

1101  PDSHAWAGNW TGRPDIGRLN AQGVLLLSAR DLGFAGAVEY LCSRLGAARR
       -*+         + -  +          * + -       -    *+    ++

1151  RLIVLDTIED WPADGPAVGD YHVYVRVRLD PAAQCAVRWP GCRELRAAVL
      +    -  --    -     -  +   + + -         + +   +- +

1201  DSSSIVGPAC FARVEASFAR LHPGAEPLRL CRQDNVRYTV STRAGPRTPV
       ***         + - *  +  +   -  +   + -  +    * +   +

1251  PLPPRAYRQR VLPTVDGCKD MARQRSALGL GDPDFDAGAA FGHRAANRWG
          +  + +      -   -   + -*      - - -       ++   +

                                                 REGION 4-->
1301  LGAPLRPVFV SCGRRGLAEL RGPEGLPAEL RAFCAAALLE PDAEAAPLVL
           +     *  **   -  +  -    -  +        -  - -

      <--REGION 5
1351  TPGAVAAAGA PPAVLWDFAP FETSVRAAAG GAVETHRPAG ASGAGAGPGE
                      -     -   +       - ++     *       -

                                                REGION 5-->1446
1401  DGDSVEIVGV RGGDGRPRGP LGPIKVEAIS DDEEAEDAGN PYLLLR
      - -* -     +  - + +          -  * ---- --          +
```

The predicted amino acid sequence (1-1446) of the PrV IE protein is shown in the single letter code with the limits of the five structural regions based on homology to the other IE proteins (Vlcek, Schwyzer and Paces, per.comm.). Acidic and basic residues are highlighted beneath the sequence (- & +) and serine residues are asterisked. The equivalent positions of the HSV-1 Vmw175 ts mutants tsK and ts1225 are shown (at residues 645 and 1092 repectively).

4. Secondary structure predictions on the Vmw175-related proteins.

Computer predictions of the potential secondary structure of the three immediate early proteins were performed using the GARNIER and PEPTIDESTRUCTURE programs, which make use of the algorithms of Chou and Fasman (1974) and Garnier et al., (1978). These programs determine the likelihood of each residue being in one of four secondary structures, H (alpha-helix), E (extended chain/ beta-pleated sheet), T (reverse turn) or C (random coil). The predictions are about 40% accurate for small globular proteins but of questionable application to larger proteins if no structural information is known. The output of the GARNIER prediction for Vmw175 and VZV 140K is included in Figures 54 and 55.

Few interesting features were revealed by these analyses. Region 1 of the HSV protein in particular was predicted to be unusually rich in random coil structure, which may reflect the limits of these types of predictive programs to identify structural features. Alternatively, the high levels of predicted random coil and GARP content in region 1 and parts of region 3 may reflect the functional unimportance of these regions.

More detailed structural analyses of the proteins in regions 2 and 4 were performed using the PEPTIDESTRUCTURE program and and profiles of the secondary structures aligned from the PLOTSTRUCTURE program (results not presented). However, because of the high amino acid identity between the three proteins the identification of apparently conserved secondary structures may merely reflect conservation of the primary structure. Whereas it was possible to identify strongly predicted and conserved structures, such as two alpha-helices in region 2 (HSV coordinates approximately 340-355 and 435-460), the significance of these was unclear. Nor did the behaviour of the insertion mutants assayed in Chapter 3 correlate with the predicted secondary structures.A conserved potential glycosylation site, NWT, at amino acid 983 of Vmw175 was also revealed in these analyses.

CHAPTER 5: DISCUSSION

The experiments described in this thesis were designed to define the regions of the HSV-1 protein Vmw175 important for functional activity. Section 1 of this Chapter summarizes the results of the mutational analysis of Vmw175 function. The important regions of the protein defined by these experiments are discussed in greater depth in Section 2, where possible models for the mechanism of Vmw175 function are also discussed. In Section 3 some possible avenues for further research into the function of Vmw175 are discussed.

1. The results of the mutational analysis of Vmw175 function.

1.1 Regions of Vmw175 involved in transcriptional regulation.

The results presented in Section 3C and summarized in Figure 17 and Tables 7 and 8 define the regions of Vmw175 important for transactivation of the promoter of the HSV-1 early gene gD (in synergy with Vmw110) and for repression of the promoter of IE gene 3 itself.

Large stretches of the protein are relatively unimportant for either function and the regions most sensitive to disruption correlate to sequences conserved between Vmw175 and VZV 140K, the corresponding transactivating protein of VZV. The region from amino acids 275 to 490 is particularly important for both repression and transactivation, whereas that from 840 to 1100 seems to be more important for transactivation than repression. These two regions of homology, B and C (Figures 5 and 49), are also conserved in the PrV IE protein IE180, confirming their evolutionary importance. The very small conserved region, A, which contains a highly serine-rich tract does not seem to be essential in these assays. Indeed a plasmid borne mutant, pD1, which lacks this region still retains significant activity in both assays. Furthermore, the region of the protein deleted in this mutant was shown to be inessential (but not unimportant) for virus growth on non-complementing cells when this mutation was recombined

back into the viral genome.

A number of previous studies have investigated how the structure of Vmw175 relates to its different activities (reviewed in Section 1D2.4). Early studies with temperature sensitive alleles gave some information on important domains of the protein; tsK was shown to be caused by a single missense mutation in homology region B (Davison et al., 1984) whilst tsD and tsT localize around region C (Preston, 1981). Different ts mutants were shown to exhibit different phenotypes and DeLuca et al. (1984) deduced that lesions mapping towards the carboxy-terminus tended to produce a later block in viral gene expression. Indeed, using a panel of plasmid-borne truncation mutants of the gene encoding Vmw175, DeLuca and Schaffer (1987) showed that the carboxy-terminal 40% and the first 90 amino acids of Vmw175 were not essential for transcriptional regulation. The results presented in this thesis are in good general agreement with these results and confirm that region 2 of Vmw175 is the most critical functional region for both repression and transactivation. However, the mutagenesis technique described in this thesis allows a more sensitive analysis of the functional regions of the protein, without producing gross changes in protein structure which may affect protein stability and function.

Insertions in most of regions 1 and 5 suggest that neither of these regions plays a critical role in the function of Vmw175. However, small deletions around the beginning of region 5 behave anomalously and abolish transactivation activity. Furthermore, whilst to some extent deletions pD2, pD4 and pD7 confirm previous reports that regions 4 and 5 can be deleted without abolishing activity in transfection assays, some insertion mutants in region 4 are severely impaired in their transactivation activity. It seems therefore that whilst region 4 is not essential for transactivation (or repression) mutations within this region can severely disrupt transactivation. This suggests that the precise configuration of region 4 is important for activity, and that this region may be playing some as yet unrecognized role in the activity of Vmw175. This will be discussed further in Section 5.2.2.

In order to assay transactivation by their truncation mutants of Vmw175 DeLuca and Schaffer (1987) used the promoter of another HSV early gene, tk, and defined similar important regions of Vmw175 to those reported here to be involved in activating the gD promoter. However, when recombined back into virus it was found that mutants encoding the first 773 amino acids were able to induce early gene expression, but did not allow DNA replication or late gene expression (DeLuca and Schaffer, 1988). Nor did these mutants over express IE3 RNA (see Section 1D2.4 and Table 3). In order to investigate the role of Vmw175 in regulating late gene expression it would be interesting to look at the ability of plasmid-borne mutants to transactivate late promoters, for example that of the VP5 gene. Because of the reported involvement of Vmw63 in regulating late gene expression (see Section 1D1.3), it would be interesting to assay the activity of the Vmw175 mutants in the presence of a plasmid encoding Vmw63. It would also be interesting to see if the mutants act similarly on heterologous non-HSV promoters and whether transactivation in the absence of Vmw110 requires the same regions as when the two proteins act in synergy.

1.2 Regions of Vmw175 involved in DNA binding

The results presented in Section 3D2 and summarized in Tables 9 and 10 show that the regions of Vmw175 important for binding to the IE3 cap site *in vitro* closely correspond to those important for transcriptional regulation in transfected cells. Although the results do not demonstrate an absolute correlation between *in vitro* DNA binding activity and either transactivation or repression, it is clear that the same general regions of the protein are involved in all three functions. In particular the integrity of region 2 is of critical importance for each activity.

DeLuca and Schaffer (1988) have used a set of viral mutants expressing truncated forms of Vmw175 to map the DNA binding domain of Vmw175. Mutants deleted for the first 90 amino acids of the protein and those removing residues downstream from 773 were essentially wild-type in their

ability to bind to a probe spanning the IE3 cap site (and in their capacity to regulate IE3 and early gene expression in the infected cell). A mutant deleted from amino acid 590 was able to form a novel DNA complex at elevated salt concentrations, but possesed no regulatory activity in the virus.

However, the mutational approach used by DeLuca and Schaffer (1988) can only investigate the importance of the carboxy-terminus of the protein, furthermore, truncated proteins with large deletions may exhibit conformational abnormalities making interpretation of results more complicated. Indeed comparing the results presented in Table 9 and 10 in this thesis it is apparent that the insertion mutants give a more consistent pattern of results than do the deletion mutants, and perhaps with the larger deletions only positive results should be considered significant. The ability of deletions pD13, pD1 and pD2F to bind to DNA shows that amino acids outside residues 229-496 are probably not directly involved in site-specific DNA binding. Those mutant polypeptides with insertions and deletions outwith this region which fail to bind DNA may be inactivated by longer range steric effects, for example disruption of protein folding.

That a relatively small portion of the Vmw175 polypeptide is required for site-specific DNA binding was confirmed by protease K digestion of the Vmw175-DNA complex. A protease resistant domain of the protein was able to bind to DNA, perhaps with even greater affinity than the wild-type protein (Section 3D1.4). This observation implies that it should be possible to express short fragments of the Vmw175 polypeptide (perhaps by <u>in vitro</u> synthesis) and more closely define the DNA binding domain.

Neither the nuclear localization signal nor the serine rich tract (homology region A) were required for DNA binding by Vmw175 deletion mutants (Section 3D2.2). In the case of proteins deficient in nuclear localization Vmw175 could be recovered from the cytoplasm and shown to bind to DNA, indicating that no nuclear modifications to the protein are essential for this activity.

The involvement of region 4 in DNA binding is unclear;

as in the transactivation assays this region of the protein is not absolutely required for activity, but some mutations in this regions can interfere with DNA binding. If this region of the protein is not directly involved in DNA binding then these mutations must be interfering with DNA binding by altering the conformation of the protein and influencing the DNA binding domain. Indeed insertions and deletions in this area of the protein did seem to cause a decrease in the mobility of protein-DNA complexes on polyacrylamide gels, as if the protein was partially denatured, but still able to bind DNA. A similar phenotype may also have been detected with the recombinant virus N9/N10, where insertion of 4 amino acids in region 5 created a polypeptide with temperature-sensitive DNA binding activity, but which formed barely detectable amounts of this "denatured" complex.

Because the regions of Vmw175 required for transcriptional activation and repression overlap with each other and with the region required for site-specific DNA binding it is not possible to directly associate site-specific binding uniquely with either regulatory activity. The weight of experimental evidence supports a role for the Vmw175 binding site at the cap site of IE gene 3 in autoregulation (Section 1D2.2) but it is still an enigma as to why the same protein sequences should apparently be necessary for both this function and transcriptional activation, which is not sequence specific. It is possible that Vmw175 possesses a second less specific DNA binding activity, perhaps encoded by the same protein sequences or alternatively elsewhere in the protein. Indeed Michael et al., (1988) have observed Vmw175 binding to the 5' transcribed non-coding regions of certain late genes which do not possess the consensus site-specific binding site. It would be interesting to investigate which regions of the protein are required for this activity, and whether these coincide with the regions (4 and 5) identified by DeLuca and Schaffer as being involved in late gene activation.

In order to investigate further the unexpected effect of mutations in the carboxy-terminal 40% of the protein to

interfere with a DNA binding specificity (apparently determined in the amino-terminal 40% of the protein) a number of ts mutant viruses, carrying lesions in the carboxy-terminal regions of Vmw175, were assayed for Vmw175-DNA binding activity. Four of these mutants were unable to bind to DNA in vitro when assayed at the NPT, a phenotype which correlated with their inability to autoregulate IE gene 3 expression in infected cells. These viruses could, however, express early gene products at the NPT. One ts mutant (ts1225, provisionally mapped to the most conserved stretch of region 4, R.D.Everett, per.comm.) was however able to bind to DNA in vitro but still overexpressed Vmw175 in the infected cell at NPT. It would be interesting to investigate whether this protein bound to the IE3 promoter in the infected cell, as this result implies that repression by Vmw175 may be more complicated than a simple "operator" binding mechanism.

Under the conditions of binding used by DeLuca and Schaffer (1988) the Vmw175-DNA complexes were salt labile when the concentration was raised from 15 to 65mM NaCl. Furthermore, they observed that some of their mutants formed novel complexes at elevated salt concentrations. In contrast to their observations, the Vmw175-IE3 cap site complex was not salt labile under the conditions of binding used in the studies reported in this thesis, and indeed the wild-type Vmw175 DNA complex was stable up to 500mM NaCl (Section 3D1.5). Furthermore, DNA binding was still salt stable at elevated temperatures, indicating a highly specific protein-DNA affinity. The nature of the novel complex formed at elevated ionic strength by proteins deleted for the carboxy-terminus of the protein is unclear (DeLuca and Schaffer, 1988). One suggestion was that this complex could represent Vmw175 monomers bound to DNA at the elevated salt concentration, whilst the wild-type, low-salt complex could represent multimers of Vmw175 bound to DNA. An alternative explantion would be that a second (host or viral) protein is absent from the complex at higher ionic strength. This rapidly migrating complex may be related to those reported in this thesis formed by wt and ts1225-Vmw175 at elevated temperature and salt concentration (Figure 44B).

The salt stability of each mutant protein-DNA complex was not investigated, but might provide a sensitive measurement of relative DNA binding activity, as proteins binding to DNA less strongly than wild-type would be dissociated from DNA at a lower ionic strength.

2. Structural Regions of Vmw175.

2.1 The role of region 2: a model for Vmw175 function.

A region of Vmw175 spanning amino acids 275-495, including a region conserved between three alphaherpesvirus IE proteins (region 2: 315-484), has been shown to be of critical importance for positive and negative transcriptional regulation in transfected cells, and site-specific DNA binding *in vitro*. Thus at least three functions are encoded by a region of the polypeptide which is of itself almost as large as the multifunctional adenovirus E1a protein. Whereas mutagenesis studies on E1a have defined separate modular domains of the protein involved in each of its activites (Section 1E3.4; Figure 9) the studies presented in this thesis suggest that the integrity of the whole of region 2 may be required for all the functions of Vmw175.

The ability of Vmw175 to bind to the ATCGTC binding site at the cap site of IE gene 3 has been shown to be required for autoregulation of this gene (DeLuca and Schaffer, 1988; Roberts *et al.*, 1988). However, the role of DNA binding in transcriptional activation is not clear. DNA binding activity may be essential to direct Vmw175 to responsive promoters, but recognition of responsive promoters is not mediated through Vmw175-specific sequences. Furthermore, Vmw175 seems to function by a similar mechanism to E1a, through the TATA box element. E1a has been shown not to bind to DNA directly.

If direct DNA binding is required for transcriptional activation by Vmw175, the results presented in this thesis would suggest that the same or overlapping protein sequences are required for this less specific promoter binding as are required to interact with ATCGTC binding sites.

The significance of ATCGTC binding sites in promoters other than that of the autoregulated IE3 gene is not known.

No functional significance has been demonstrated for the sites upstream of the IE1 and IE2 promoters. The site upstream from the gD promoter may contribute to the activation of this promoter, at least in some conditions (Tedder and Pizer, 1988) but does not seem to be required for activation of this promoter (Everett, 1983). Studies are ongoing to determine the significance of this binding site in the viral life-cycle (Everett, R.D. and Pizer, L., per.comm.). It is possible that the role of this site is indirect and its contribution is to access Vmw175 to the general region of the gD promoter, without being essential for activation. The positioning of the ATCGTC Vmw175 binding site over the transcriptional start site of the IE3 gene may well be critical for its role in repressing IE3 transcription.

A distinct "activating" domain, separable from a DNA binding domain has not been defined for Vmw175. This contrasts with the adenovirus E1a protein where activation function is specified by a short 49 amino acid region, which can function as a transcriptional activator independently from the rest of the polypeptide. The E1a activating region has a zinc-binding finger motif, probably involved in interacting with cellular proteins to mediate activation. Region 2 of Vmw175 has no such recognizable amino acid structures, nor have these studies identified an "acidic" activating domain in this region involved in activation. It is possible, however, that regions involved in activation lie outwith region 2 and have not been identified by these studies. It will be important to determine whether a polypeptide spanning region 2, but lacking all the potential acidic domains in other regions of the protein, can still function as a transcriptional regulator. Although there is no definable "activating region" in Vmw175 that resembles the structure of previously recognized activating regions, it is quite possible that such an activating region in Vmw175 represents a novel type of structure.

Taking into consideration the known properties of Vmw175 and recent studies on the function of the PrV IE180 protein (Section 1D2.1), it is possible to propose a simple functional model that could account for the dual role of

Vmw175 in transcriptional activation and repression. The model proposes that Vmw175 interacts simultaneously both with DNA and with host transcription factors, such as the TATA box binding factor TFIID, in order to potentiate production of a functional stabilized transcription complex. If the DNA binding region of Vmw175 encounters a very strong binding site sequence, such as that at the IE3 cap site, then the high affinity constant of the DNA/Vmw175 complex could effectively immobilize the transcriptional machinery. If the DNA sequence is less strongly bound by Vmw175, then the stabilized transcription complex would be free to proceed. Note that the proposed site of interaction with the host transcription factors could also map in region 2. This model is consistent with the importance of the TATA/cap region for activation of the tk promoter (Coen et al., 1986) and the binding of Vmw175 to (non-consensus and possibly weak) sites in the 5' untranslated regions of other activated HSV genes (Michael et al., 1988).

2.2 The role of region 4

The carboxy-terminal 40% of Vmw175, including the region conserved between three alphaherpesvirus IE proteins (region 4: residues 797-1224), is essential for growth in infected cells (DeLuca and Schaffer, 1988). Viruses with deletion or ts mutations in this portion of the protein are able to induce early gene expression but not viral DNA replication, nor full late gene expression (Preston, 1981; DeLuca et al., 1984; DeLuca and Schaffer, 1988; this thesis). It is not known whether this region of the protein is simply important for the conformational stability of the whole protein, or whether it directs a novel uncharacterized activity.

This region of the protein does not appear to be essential in transfection assays for transcriptional regulation and DNA binding activity (DeLuca and Schaffer, 1987, 1988; this thesis) although some insertion and deletion mutations in this region do severely impair transcriptional regulation and DNA binding activity. Therefore the carboxy-terminal domains of Vmw175 do not seem to have a direct role in these functions, but can interfere with these activities which are probably specified by region

2 of the protein.

Vmw175 is not thought to have a direct role in viral DNA replication. Plasmid based replication assays require the presence of Vmw175, but apparently only to activate expression of the replication genes (Challberg, 1986; Wu et al., 1988; R. Heibronn and H. zur Hausen, 1988, abstract 226 at the 13th International Herpesvirus Workshop). It is possible that Vmw175 has a role in the viral life-cycle to activate expression of the replication genes, and that perhaps the mechanism of this activation is distinct from the general activation of other early genes. This activation could be dependent on region 4 of the protein. Alternatively, mutant Vmw175 with ts or deletion lesions in region 4 may interfere with the expression of these replication genes, or even interfere directly with replication activity.

It has been suggested that mutations in region 4 of Vmw175 may interfere with the sub-nuclear localization of Vmw175 or other proteins (DeLuca and Schaffer 1987, 1988), but they could also interfere with Vmw175 function in number of other ways. Disruption of region 4 may cause severe conformational abnormalities which affect the activity of other regions of the protein. If region 4 interacts with region 2 of the protein a perturbation in the structure of region 4 may be more deleterious than loss of the whole region. This might explain why the large out of frame deletions pD2 and pD7 have more activity than their in-frame daughters pD2F and pD7F. Region 4 could in fact be involved in controlling or modulating the activity of region 2, in a similar fashion to the hormone binding domains of the steroid receptors (Section 1E3.2), and deletion of region 4 alleviates region 2 from this control. Region 4 might also be involved in the interaction of Vmw175 with other cellular or viral factors, such as Vmw110, involved in transcriptional activation. If activation of late gene expression occurs by a separate mechanism to activation of early genes, region 4 may be involved in this function in conjunction with Vmw63, perhaps via a direct interaction with this protein.

2.3 The role of the serine-rich tract.

Vmw175 contains a serine-rich tract followed by a highly acidic run (residues 176-206) which is conserved between the three alphaherpesvirus IE proteins (Figure 50). Deletion of this sequence does not abolish transcriptional regulation by Vmw175 in transfection assays nor DNA binding *in vitro*, and this region is not essential for virus growth on a non-complementing cell-line.

This serine-rich tract is probably the major phosphorylation site of Vmw175 (DeLuca and Schaffer, 1988) although threonine residues elsewhere in the protein are also phosphorylated (Faber and Wilcox, 1986b). The phosphorylation state of Vmw175 produced by the recombinant virus I15HBC should be investigated to confirm the involvement of this region in phosphorylation. If this region is the major phosphorylation site for Vmw175, this modification would not appear to be essential for Vmw175 activity. Although modification by phosphorylation has been reported to be a major control mechanism for several proteins involved in transcriptional control (see Section 1E3.5), the phosphorylation state of the adenovirus E1a protein does not affect its transactivation and repression activity (Richter *et al.*, 1988).

Three major different electrophoretic forms of Vmw175 can be distinguished which differ in post-translational modification of the protein, including phosphorylation (Wilcox *et al.*, 1980). These different forms of the Vmw175 bind with different affinities to DNA probes which do not include an ATCGTC consensus (Michael *et al.*, 1988). It would therefore be interesting to investigate DNA binding by this deleted protein to DNA probes other than the IE3 cap site probe used in the studies reported in this thesis.

The phenotype of the recombinant virus I15HBC which is deleted for amino acids 162-229 of Vmw175 suggests that this region does contribute to Vmw175 function. This virus forms small, syncitial plaques and may have a high particle to pfu ratio; it is defective in inducing viral DNA replication and grows to a low titre. These phenotypes could be caused by a failure to turn on expression of certain early and late genes to maximal levels.

2.4 The nuclear localization signal.

Immunofluorescent staining of cells transfected with deletions of IE gene 3 defined the sequences required for nuclear localization of Vmw175 to within amino acids 682-774. This region contains a good candidate for a nuclear localization signal consisting of four consecutive basic amino acids at residue 727 (Figure 52). This site is identically conserved in VZV 140K and similar sequences are found in the corresponding region of PrV IE180. This site resembles signals in SV40 T antigen and Ela which are involved in nuclear localization (Kalderon et al., 1984; Krippl et al., 1985). A similar basic string in Vmw110 seems to be involved in nuclear localization (Everett, 1988a).

Nuclear localization does not appear to be prerequisite for DNA binding activity as proteins deficient in nuclear localization can be isolated from the cytoplasm of transfected cells and shown to bind to DNA (DeLuca and Schaffer, 1988; this thesis). Proteins deleted for the nuclear localization signal must still accumulate in the nucleus to a certain extent to allow a degree of transcriptional regulation, although not at levels sufficient to allow detection by immunofluorescence (see Table 8). That these mutants are less impaired for transactivation than repression suggests that these two activities may function with different stoichiometries.

Temperature sensitive mutations in other regions of the protein have also been reported to disrupt the nuclear localization of Vmw175 (Preston, 1979b) and to interfere with the localization of some (but not all) viral proteins including Vmw110 and the major DNA binding protein (Knipe and Smith, 1986). This suggests that Vmw175 may form a complex with these proteins which localizes as unit to the nucleus.

3. Future research prospects.

As a result of the research presented in this thesis and by other workers much is now known about the functionally important regions of Vmw175 and how this protein may function in regulating transcription in the viral life-cycle. However, there are still large gaps in our knowledge. The three most important areas for further research on Vmw175 are: (i) The functional role of the carboxy-terminal 40% of Vmw175, which is essential *in vivo* but has not as yet been assigned a function. (ii) The role of DNA binding in transactivation and the significance of binding to DNA sites lacking ATCGTC elements. (iii) The biochemical manner by which Vmw175 interacts with the host transcriptional machinery to activate and repress transcription.

The panel of insertion and deletion mutatations within the Vmw175 coding sequence described in this thesis will provide an important tool to continue these studies in this Institute.

To date only the activation of early HSV promoters by mutant Vmw175 polypeptides has been studied in detail. It would be illuminating to investigate whether the same protein regions were involved in the activation of late gene promoters, and whether the regions important for activation vary when assayed in the presence or absence of Vmw110 and Vmw63.

Techniques similar to those described in this thesis could be used to investigate DNA binding to probes not containing the consensus ATCGTC binding site, and whether the same domains of Vmw175 are involved in binding to these sites as are involved in binding to the IE3 cap site. The significance of the *in vitro* DNA binding assay to the situation in the infected cell could also be investigated. This could be done by "footprinting" protein binding sites on the viral genome, by treating infected cells with DMS and performing "genomic sequencing" on the viral DNA. The significance of the DNA binding activities of Vmw175 to other ATCGTC or non-ATCGTC sequences could then be determined. It would be particularly interesting to see if *ts*1225-Vmw175 binds to the IE3 cap site *in vivo* as this

virus fails to autoregulate IE3 expression.

The characterization of the three viral mutants whose construction is described in this thesis should be continued. The results presented in this thesis are of a preliminary nature and should be confirmed. In particular the phenotype of I15HBC should be analysed, including whether the Vmw175 polypeptide expressed by this virus, which lacks the serine-rich tract, is phosphorylated. Because the phenotype of this mutant is so interesting and somewhat unexpected it would be prudent to subclone the mutant IE3 allele from this virus in order to confirm its DNA lesion.

A more detailed analysis of the DNA binding domain of Vmw175 should prove interesting, and could resolve the sequences required for site-specific DNA binding from those required for transcriptional activation. This could be pursued by expressing peptide fragments of Vmw175 *in vitro* in order to identify the short DNA binding domain corresponding to the protease resistant domain "p" defined in this thesis. This would then allow the specific amino acids involved in DNA binding to be investigated by site-directed mutagenesis.

Studies upon the biochemical action of the PrV IE180 protein are obviously well advanced (Abmayr *et al.*, 1988; Workman *et al.*, 1988) and it probably would not be fruitful to attempt to duplicate these studies with Vmw175 at this stage.

REFERENCES

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

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

ACE, C.I., DALRYMPLE, M.A., RAMSAY, F.H., PRESTON, V.G., and PRESTON, C.M. (1988). Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65. J. Gen. Virol. 69, 2595-2605.

ACE, C.I., McKEE, T.A., RYAN, J.M., CAMERON, J.M., and PRESTON, C.M. (1989). Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate early expression. Submitted.

ACKERMAN, M., BRAUN, D.K., PEREIRA, L. and ROIZMAN, B. (1984). Characterization of the herpes simplex virus 1 alpha proteins 0, 4 and 27 with monoclonal antibodies. J. Virol. 52, 108-118.

ADDISON, C., RIXON, F.J., PALFREYMAN, J.W., O'HARA, M., PRESTON, V.G. (1984). Characterisation of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology 138, 246-260.

ADLER, S., WATERMAN, M.L., HE, X., and ROSENFELD, M.G. (1988). Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA binding domain. Cell 52, 685-695.

AHLERS, S.E. and FELDMAN, L.T. (1987a). Effects of a temperature-sensitive mutation in the immediate-early gene of pseudorabies virus on class II and class III gene transcription. J. Virol. 61, 1103-1107.

AHLERS, S.E. and FELDMAN, L.T. (1987b). Immediate-early protein of pseudorabies virus is not continuously required to reinitiate transcription of induced genes. J. Virol. 61, 1258-1260.

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

ANGEL, P., CHIU, R., STEIN, B., IMBRA, R.J., RAHMSDORF, H.J., JONAT, C., HERRLICH, P. and KARIN, M. (1987). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49, 729-739.

ANGEL, P., ALLEGRETTO, E.A., OKINO, S., HATTORI, K., BOYLE, W.J., HUNTER, T. and KARIN, M. (1988). The jun oncogene encodes a sequence specific trans-activator similar to AP-1. Nature 332, 166-171.

ARSENAKIS, M., CAMPADELLI-FIUME, G. and ROIZMAN, B. (1988). Regulation of glycoprotein D synthesis: does alpha 4, the major regulatory protein of herpes simplex virus 1, regulate late genes both positively and negatively? J. Virol. 62, 148-158.

BAER, R., BANKIER, A.T., BIGGIN, M.D., DEININGER, P.L., FARRELL, P.J., GIBSON, T.J., HATFULL, G., HUDSON, G.S., SATCHWELL, S.C., SEGUIN, C., TUFFNELL, P.S. and BARRELL, B.G. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310, 207-211.

BAEUERLE, P.A., and BALTIMORE, D. (1988). I_kB: A specific inhibitor of the NF-$_k$B transcription factor. Science 242, 540-546.
BARBERIS, A., SUPERTI-FURGA, G. and BUSSLINGER, M. (1987). Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. Cell 50, 347-359.
BARINGER, J.R. and SWOVELAND, P. (1973). Recovery of herpes simplex virus from human trigeminal ganglions. N. Engl. J. Med. 288, 648-650.
BATTERSON, W., FURLONG, D. and ROIZMAN, B. (1983). Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant, defective in release of viral DNA and in other stages of the viral reproductive cycle. J. Virol. 45, 397-407.
BATTERSON, W., and ROIZMAN, B. (1983). Characterisation of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J. Virol. 46, 371-377.
BAYLISS, G.J., MARSDEN, H.M., and HAY, J. (1975). Herpes simplex virus proteins: DNA-binding proteins in infected cells and in the virus structure. Virology 68, 124-134.
BEARD, P., FABER, S., WILCOX, K.W., and PIZER, L.I. (1986). Herpes simplex virus immediate early infected cell polypeptide 4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA 83, 4016-4020.
BECKER, B., GLASS, B., SHMID, W., STRAHLE, U. and SCHUTZ, G. (1986). In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. Nature 324, 686-688.
BEN-PORAT, T. and KAPLAN, A.S. (1962). The chemical composition of herpes simplex and pseudorabies viruses. Virology 12, 261-266.
BERG, J.M. (1986). Potential metal-binding domains in nucleic acid binding proteins. Science 232, 485-487.
BLAIR, E.D., BLAIR, C.C. and WAGNER, E.K. (1987). Herpes simplex virus virion stimulatory protein mRNA leader contains sequence elements which increase both virus-induced transcription and mRNA stability. J. Virol. 61, 2499-2508.
BOHMANN, D., BOS, T.J., ADMON, A., NISHIMURA, T., VOGT, P.K. and TJIAN, R. (1987b). Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science 238, 1386-1392.
BOHMANN, D., KELLER, W., DALE, T., SCHOLER, H.R., TEBB, G. and MATTAI, I. (1987a). A transcription factor which binds to the enhancers of SV40, immunoglobin heavy chain and U2 snRNA genes. Nature 325, 268-272.
BOND, V.C., PERSON, S. and WARNER, S.C. (1982). The isolation and characterization of mutants of herpes simplex virus type 1 that induce cell fusion. J. Gen. Virol. 61, 245-254.
BORELLI, E., HEN, R. and CHAMBON, P. (1984). Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature 312, 608-612.
BOS, T.J., BOHMANN, D., TSUCHIE, H., TJIAN, R. and VOGT, P.K. (1988). V-jun encodes a nuclear protein with enhancer binding properties of AP-1. Cell 52, 705-712.
BOYER, H.W. and ROULLAND-DUSSOIX, D. (1969). A complementation analysis of the restriction modification of DNA in Escherichia coli. J. Mol. Biol. 41, 459-472.
BRADFORD, M.M. (1976). A rapid and sensitive method for the

quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.
BRENT, R. and PTASHNE, M. (1985). A eukaryotic transcription activator bearing the DNA specificity of a prokaryotic repressor. Cell 43, 729-736.
BRIGGS, M.R., KADONAGA, J.T., BELL, S.P. and TJIAN, R. (1986). Purification and biochemical characterization of the promoter-specific transcription factor, Spl. Science 234, 47-52.
BROWN, S.M. and HARLAND, J. (1987). Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11 and US12 and two in which R_S has been extended by 6kb to 0.91 map units with the loss of U_S sequences between 0.94 and the U_S/TR_S junction. J. Gen. Virol. 68, 1-18.
BROWN, S.M., HARLAND, J. and SUBAK-SHARPE, J.H. (1984). Isolation of restriction endonuclease site deletion mutants of herpes simplex virus. J. Gen. Virol. 65, 1053-1068.
BROWN, S.M., RITCHIE, D.A. and SUBAK-SHARPE, J.H. (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. Gen. Virol. 18, 329-346.
BZIK, D.J., FOX, B.A., DeLUCA, N.A. and PERSON, S. (1984). Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology, 133, 301-314.
BZIK, D.J., and PRESTON, C.M. (1986). Analysis of DNA sequences which regulate the transcription of herpes simplex virus immediate early gene 3: DNA sequences required for enhancer-like activity and response to trans-activation by a virion polypeptide. Nucleic Acids Res. 14, 929-943.
CAI, W., GU, B. and PERSON, P. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. 62, 2596-2604.
CAMERON, I.R., PARK, M., DUTIA, B.M., ORR, A. and MACNAB, J.C.M. (1985). Herpes simplex virus sequences involved in the initiation of oncogenic morphological transformation of rat cells are not required for maintenance of the transformed state. J. Gen. Virol. 66, 517-527.
CAMPADELLI-FIUME, G., ARSENAKIS, M., FARALEGOLI, F. and ROIZMAN, B. (1988a). Entry of herpes simplex virus in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. J. Virol. 62, 159-167.
CAMPADELLI-FIUME, G., AVITABILE, E., FINI, S., STIRPE, D., ARSENAKIS, M. and ROIZMAN, B. (1988b). Herpes simplex virus glycoprotein D is sufficient to induce spontaneous pH independent fusion in a cell line that constitutively expresses the glycoprotein. Virology 166, 598-602.
CAMPBELL, M.E.M., PALFREYMAN, J.W., and PRESTON, C.M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. 180, 1-19.
CAMPBELL, M.E.M. and PRESTON, C.M. (1987). DNA sequences which regulate the expression of the pseudorabies virus major immediate early gene. Virology 157, 307-316.
CARADONNA, S.J., WORRAD, D. and LORETTE, R. (1987). Isloation of a herpes simplex virus cDNA encoding the DNA

repair enzyme uracil-DNA glycosylase. J. Virol. 61, 3040-3047.
CARMICHAEL, E.P., KOSOVSKY, M.J. and Weller, S.K. (1988) Isolation and characterization of herpes simplex virus type 1 host range mutants defective in viral DNA synthesis. J. Virol. 62, 91-99.
CHALLBERG, M.D. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. USA 83, 9094-9098.
CHIU, R., BOYLE, W.J., MEEK, J., SMEAL, T., HUNTER, T. and KARIN, M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54, 541-552.
CHODOSH, L.A., BALDWIN, A.S., CARTHEW, R.W. and SHARP, P.A. (1988a). Human CCAAT-binding proteins have heterologous subunits. Cell 53, 11-24.
CHODOSH, L.A., OLESEN, J., HAHN, S., BALDWIN, A.S., GUARENTE, L. and SHARP, P.A. (1988b). A yeast and a human CCAAT-binding protein have heterologous subunits that are functionally interchangeable. Cell 53, 25-35.
CHOU, P.Y. and FASMAN, G.D. (1974). Prediction of protein conformation. Biochemistry, 13, 222-245.
CLEMENTS, J.B., McLAUGHLAN, J., and McGEOCH, D.J. (1979). Orientation of herpes simplex virus type 1 immediate-early mRNAs. Nucleic Acids Res. 7, 77-91.
CLEMENTS, J.B., WATSON, R.J., and WILKIE, N.M. (1977). Temporal regulation of herpes simplex virus type 1 transcription; location of transcripts on the viral genome. Cell 12, 275-285.
COEN, D., WEINHEIMER, S.P., and McKNIGHT, S.L. (1986). A genetic approach to promoter recognition during trans-induction of viral gene expression. Science 234, 53-59.
COHEN, G.H., PONCE de LEON, M., DIGGLEMAN, H. LAWRENCE, W.C., VERNON, S.K. and EISENBERG, R.J. (1980). Structural analysis of the capsid polypeptides of herpes simplex virus type 1 and 2. J. Virol. 34, 521-531.
CONLEY, A.J., KNIPE, D.M., JONES, P.C., and ROIZMAN, B. (1981). Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. J. Virol. 37, 191-206.
COOK, M.L., BASTONE, V.B. and STEVENS, J.G. (1974). Evidence that neurons harbour latent herpes simplex virus. Infection and Immunity, 9, 946-951.
CORDEN, J.L., CADENA, D.L., AHEARN, J.M. and DAHMUS, M.E. (1985). A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II. Proc. Natl. Acad. Sci. USA 82, 7934-7938.
CORDINGLEY, M.G., CAMPBELL, M.E.M., and PRESTON, C.M. (1983). Functional analysis of a herpes simplex virus type 1 promoter: identification of far-upstream regulatory sequences. Nucleic Acids Res. 11, 2347-2365.
CORDINGLEY, M.G., REGEL, A.T., and HAGER, G.L. (1987). Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary tumor virus in vitro. Cell 48, 261-270.
CORSALO, C.M., and PEARSON, M.L. (1981). Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. Somatic cell genetics 7, 603-616.
COSTA, R.H., DRAPER, K.G., DEVI-RAO, G., THOMPSON, R.L., and

WAGNER, E.K. (1985). Virus-induced modification of the host cell is required for expression of the bacterial chloramphenicol acetyltransferase gene controlled by a late herpes simplex virus promoter (VP5). J. Virol. 56, 19-30.
COSTANZO, F., CAMPADELLI-FIUME, G., FOA-TOMASI, L., and CASSAI, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. J. Virol. 21, 996-1001.
COUREY, A.J. and TJIAN, R. (1988). Analysis of Spl in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55, 887-898.
COURTNEY, R.J., SCHAFFER, P.A. and POWELL, K.L. (1976). Synthesis of virus-specific polypeptides by temperature-sensitive mutants of herpes simplex virus type 1. Virology 75, 306-318.
CRUTE, J.J., MOCARSKI, E.S. and LEHMAN, I.R. (1988). A DNA helicase induced by herpes simplex virus type 1. Nucleic Acids Res. 16, 6585-6595.
CULP, J.S., WEBSTER, L.C., FRIEDMAN, D.J., SMITH, C.L., HUANG, W.-J., WU, F.Y.-H., ROSENBERG, M. and RICCIARDI, R.P. (1988). The 289-amino acid Ela protein of adenovirus binds zinc in a region that is important for transactivation. Proc. Natl. Acad. Sci. USA 85, 6450-6454.
CURRAN, T. and FRANZA, B.R. (1988). Fos and Jun: the AP-1 connection. Cell 55, 395-397.
DALRYMPLE, M.A., McGEOCH, D.J., DAVISON, A.J. and PRESTON, C.M. (1985). DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate early promoters. Nucl. Acids Res. 13, 7865-7879.
DAVIDSON, I., FROMENTAL, C., AUGEREAU, P., WILDEMAN, A., ZENKE, M and CHAMBON, P. (1986). Cell-type specific protein binding to the enhancer of simian virus 40 in nuclear extracts. Nature 323, 544-548.
DAVIDSON, I., and STOW, N.D. (1985). Expression of an immediate-early polypeptide and activation of a viral origin of DNA replication in cells containing a fragment of herpes simplex virus DNA. Virology 141, 77-88.
DAVIDSON, I., XIAO, J.H., ROSALES, R., STAUB, A. and CHAMBON, P. (1988). The HeLa cell protein TEF-1 binds specifically and cooperatively to two SV40 enhancer motifs of unrelated sequence. Cell 54, 931-943.
DAVISON, A.J. (1981). Detailed structural aspects of the HSV genome. PhD thesis, University of Glasgow.
DAVISON, A.J., and McGEOCH, D.J. (1986). Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and Varicella-Zoster virus. J. Gen. Virol. 67, 597-611.
DAVISON, A.J. and SCOTT, J.E. (1986). The complete sequence of varicella zoster virus. J. Gen. Virol. 67, 1759-1816.
DAVISON, A.J. and TAYLOR, P. (1987). Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. 68, 1067-1079.
DAVISON, A.J. and WILKIE, N.M. (1981). Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. J. Gen. Virol. 55, 315-331.
DAVISON, A.J. and WILKIE, N.M. (1983a). Inversion of the two segments of the herpes simplex virus genome in intertypic recombinants. J. Gen. Virol. 64, 1-18.
DAVISON, A.J., and WILKIE, N.M. (1983). Location and orientation of homologous sequences in the genomes of five

herpesviruses. J. Gen. Virol. 64, 1927-1942.
DAVISON, B.L., EGLY, J-M., MULVIHILL, E.R., and CHAMBON, P. (1983). Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature (London) 301, 680-686.
DAVISON, M.-J., PRESTON, V.G., and McGEOCH, D.J. (1984). Determination of the sequence alteration in the DNA of the herpes simplex virus type 1 temperature-sensitive mutant tsK. J. Gen. Virol. 65, 859-863.
DEB, S. and DOELBERG, M. (1988). A 67-base-pair segment from the Ori-S Region of herpes simplex virus type 1 encodes origin function. J. Virol. 67, 2516-2519.
DEISS, L.P. and FRENKEL, N. (1986). The herpes simplex amplicon: cleavage of concatameric DNA is linked to packaging and involves amplification of the terminally reiterated a sequence. J. Virol. 57, 933-941.
DEISS, L.P., CHOU, J. and FRENKEL, N. (1986). Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. 59, 605-618.
De BRUYN KOPS, A. and KNIPE, D.M. (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell 55, 857-868.
DeLUCA, N.A., BZIK, D.J., BOND, V.C., PERSON, S. and SNIPES, W. (1982). Nucleotide sequences of herpes simpex virus type 1 (HSV-1) affecting virus entry, cell fusion and production of glycoprotein gB (VP7). Virology 122, 411-423.
DeLUCA, N.A., COURTNEY, M.A., and SCHAFFER, P.A. (1984). Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. J. Virol. 52, 767-776.
DeLUCA, N.A., McCARTHY, A.M., and SCHAFFER, P.A. (1985). Isolation and characterisation of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. 56, 558-570.
DeLUCA, N.A., and SCHAFFER, P.A. (1985). Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. 5, 1997-2008.
DeLUCA, N.A., and SCHAFFER, P.A. (1987). Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense polypeptides. Nucleic Acids Res. 15, 4491-4511.
DeLUCA, N.A., and SCHAFFER, P.A. (1988). Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. 62, 732-743.
DENIS, M., POELLINGER, L., WIKSTOM, A.-C. and GUSTAFSSON, J.-A. (1988). Requirement of hormone for thermal conversion of the glucocorticoid receptor to a DNA-binding state. Nature 333, 686-688.
DENNIS, D., and SMILEY, J.R. (1984). Transactivation of a late herpes simplex virus promoter. Mol. Cell. Biol. 4, 544-551.
DIGNAM, J.D., LEBOVITZ, R.M., and ROEDER, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11, 1475-1489.
DIXON, R.A.F., and SCHAFFER, P.A. (1980). Fine structure mapping and functional analysis of temperature-sensitive

mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. 36, 189-203.
DORSKY, D.I. and CRUMPACKER, C.S. (1988). Expression of herpes simplex virus type 1 DNA polymerase gene by in vitro translation and effects of gene deletions on activity. J. Virol. 62, 3224-3232.
DUSH, M.K., SIKELA, J.M., KHAN, S.A., TISCHFIELD, J.A., and STAMBROOK, P.J. (1985). Nucleotide sequence and organisation of the mouse adenine phosphoribosyltransferase gene: Presence of a coding region common to animal and bacterial phosphoribosyltransferases that has a variable intron/exon arrangement. Proc. Natl. Acad. Sci. USA 82, 2731-2735.
EFSTATHIOU, S., MINSON, A.C., FIELD, H.J., ANDERSON, J.R. and WILDY, P. (1986). Detection of herpes simplex virus-specific sequences in latently infected mice and humans. J. Virol. 57, 446-455.
EGAN, C., JELSMA, T.N., HOWE, J.A. BAYLEY, S.T., FERGUSON, B. and BRANTON, P.E. (1988). Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. Mol. Cell. Biol. 8, 3955-3959.
EISENBERG, S.P., COEN, D.M., and McKNIGHT, S.L. (1985). Promoter domains required for expression of plasmid-borne copies of the herpes simplex virus thymidine kinase gene in virus-infected mouse fibroblasts and microinjected frog oocytes. Mol. Cell. Biol. 5, 1940-1947.
ELIAS, P., O'DONNELL, M.E., MOCARSKI, E.S. and LEHMAN, I.R. (1986). A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 83, 6322-6326.
ELIAS, P. and LEHMAN, I.R. (1988). Interaction of origin binding protein with an origin of replication of herpes simplex virus 1. Proc. Natl. Acad. Sci. USA 85, 2959-2963.
El KAREH, A., MURPHY, J.M., FICHTER, T., EFSTRATIADIS, A., and SILVERSTEIN, S. (1985). "Transactivation" control signals in the promoter of the herpesvirus thymidine kinase gene. Proc. Natl. Acad. Sci. USA 82, 1002-1006.
EPSTEIN, M.A. (1962). Observations on the fine structure of mature herpes simplex virus and on the composition of its nucleoid. J. Exp. Med. 115, 1-9.
EVANS, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science 240, 889-895.
EVERETT, R.D. (1983). DNA sequence elements required for regulated expression of the HSV-1 glycoprotein D gene lie within 83 bp of the RNA capsites. Nucleic Acids Res. 11, 6647-6666.
EVERETT, R.D. (1984a). A detailed analysis of an HSV-1 early promoter: sequences involved in trans-activation by viral immediate-early gene products are not early-gene specific. Nucleic Acids Res. 12, 3037-3056.
EVERETT, R.D. (1984b). Transactivation of transcription by herpes virus products; requirements for two HSV-1 immediate early polypeptides for maximum activity. EMBO J. 3, 3135-3141.
EVERETT, R.D. (1985). Activation of cellular promoters during herpes virus infection of biochemically transformed cells. EMBO J. 4, 1973-1980.
EVERETT, R.D. (1986). The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1, 2 and 3 can activate gene expression in trans. J. Gen. Virol. 67, 2507-2513.
EVERETT, R.D. (1987a). The regulation of transcription of

viral and celular genes by herpesvirus immediate-early gene products. Anticancer Research 7, 589-604.
EVERETT, R.D. (1987b). A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. EMBO J. 6, 2069-2076.
EVERETT, R.D. (1988a). Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J. Mol. Biol. 202, 87-96.
EVERETT, R.D. (1988b). Promoter sequence and cell type can dramatically affect the efficiency of transcriptional activation induced by herpes simplex virus type 1 and its immediate-early products Vmw175 and Vmw110. J. Mol. Biol. 203, 739-751.
EVERETT, R.D. (1989). Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate-early gene 1. Submitted.
FABER, S.W., and WILCOX, K.W. (1986a). Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res. 14, 6067-6083.
FABER, S.W., and WILCOX, K.W. (1986b). Characterisation of a herpes simplex virus regulatory protein: Aggregation and phosphorylation of a temperature sensitive variant of ICP4. Archives of Virology 91, 297-312.
FABER, S.W., and WILCOX, K.W. (1988). Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. Nucleic Acids Res. 16, 555-570.
FEINBERG, A.P. and VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry, 132, 6-13.
FELSER, J.M., STRAUS, S.E., and OSTROVE, J.M. (1987). Varicella-Zoster virus complements herpes simplex virus type 1 temperature-sensitive mutants. J. Virol. 61, 225-228.
FELSER, J.M., KINCHINGTON, P.R., INCHAUPSE, G., STRAUSE, S.E. and OSTROVE, J.M. (1988). Cell-lines containing Varicella-Zoster virus open reading frame 62 and expressing th "IE" 175 protein complement ICP4 mutants of the herpes simplex virus type 1. J. Virol. 62, 2076-2082.
FENWICK, M.L. and OWEN, S.A. (1988). On the control of immediate early (alpha) mRNA survival in cells infected with herpes simplex virus. J. Gen. Virol. 69, 2869-2879.
FENWICK, M.L. and WALKER, M.J. (1979). Phosphorylation of a ribosomal protein and of virus-specific proteins in cells infected with herpes simplex virus. J. Gen. Virol. 45, 397-405.
FENWICK, M.L. (1984). The effects of herpes viruses on cellular macromolecular synthesis, in: "Comprehensive Virology" vol. 19, pp359-390, H. Fraenkel-Conrat and R.R. Wagner (eds.) Plenum Press, N.Y.
FERGUSON, B., KRIPPL, B., ANDRISANI, O., JONES, N., WESTPHAL, H. and ROSENBERG, M. (1985). E1A 13S and12S mRNA products made in Escherichia coli both function as nucleus-localized transcription activators but do not directly bind DNA. Mol. Cell. Biol. 5, 2653-2661.
FILION, M., SKUP, D. and SUH, M. (1988). Specific induction of cellular gene transcription in herpes simplex virus type 2-transformed cells. J. Gen. Virol. 69, 2011-2019.
FIRE, A., SAMUELS, M. and SHARP, P.A. (1982). Interactions between RNA polymerase II, factors, and template leading

to accurate transcription. J. Biol. Chem. 259, 2509-2516.
FISCHER, J.A., GINIGER, E., MANIATIS, T. and PTASHNE, M. (1988). GAL4 activates transcription in Drosophila. Nature 332, 853-856.
FLETCHER, C., HEINTZ, N. and ROEDER, R.G. (1987). Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell 51, 773-781.
FRANKEL, A.D and PABO, C.O. (1988). Fingering too many proteins. Cell 53, 675.
FREEMAN, J.M., and POWELL, K.L. (1982). DNA-binding properties of a herpes simplex virus immediate early protein. J. Virol. 44, 1084-1087.
FRIEDMAN, A.D., TRIEZENBERG, S.J. and McKNIGHT, S.L. (1988). Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus. Nature 335, 452-254.
FROMENTAL, C., KANNO, M., NOMIYAMA, H. and CHAMBON, P. (1988). Cooperativity and hierarchical levles of functional organization in the SV40 enhancer. Cell 54, 943-953.
GAFFNEY, D.F., McLAUCHALN, J., LINDSAY, J., and CLEMENTS, J.B. (1985). A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. Nucleic Acids Res. 13, 7847-7863.
GALLOWAY, D.A. and McDOUGALL, J.K. (1983). The oncogenic potential of herpes simplex viruses: evidence for a "hit and run" mechanism. Nature 302, 21-24.
GALLOWAY, D.A., NELSON, J.A. and McDOUGALL, J.K. (1984). Small fragments of herpesvirus DNA with transforming activity contain insertion-like structures. Proc. Natl. Acad. Sci. USA 81, 4736-4740.
GARNER, M.M. and REVZIN, A. (1981) A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system, Nucleic Acids Res. 9, 3047-3060.
GARNIER,J., OSGUTHORPE, D.J. and B. ROBSON (1978). Analysis of the accuracy and implications of simple methods for ptedicting the secondary structure of globular proteins. J. Mol. Biol. 120, 97-120.
GELB, L.D. (1985). Varicella-zoster virus, in: "Virology", pp591-627, B.N. Fields (ed.), Raven Press, N.Y.
GELMAN, I.H., and SILVERSTEIN, S. (1985). Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA 82, 5265-5269.
GELMAN, I., and SILVERSTEIN, S. (1986). Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate-early gene products. J. Mol. Biol. 191, 395-409.
GELMAN, I.H., and SILVERSTEIN, S. (1987a). Herpes simplex virus immediate-early promoters are responsive to virus and cell trans-acting factors. J. Virol. 61, 2286-2296.
GELMAN, I.H., and SILVERSTEIN, S. (1987b). Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the virus transcriptional activators. J. Virol. 61, 3167-3172.
GERSTER, T. and ROEDER, R.G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl.

Acad. Sci. USA 85, 6347-6351.
GIBSON, M.G., and SPEAR, P.G. (1983). Insertion mutants of herpes simplex virus have a duplication of the glycoprotein D gene and express two different forms of glycoprotein D. J. Virol. 48, 396-404.
GIGUERE, V., HOLLENBERG, S.M., ROSENFELD, M.G. and EVANS, R.M. (1986). Functional domains of the human glucocorticoid receptor. Cell 46, 645-652.
GILL, G. and PTASHNE, M. (1988). Negative effect of the transcriptional activator GAL4. Nature 334, 721-724.
GILL, G. and PTASHNE, M. (1987). Mutants of the GAL4 protein altered in an activation function. Cell 51, 121-126.
GINIGER, E. and PTASHNE, M. (1987). Transcription in yeast activated by a putative amphipathic alpha-helix linked to a DNA binding unit. Nature 330, 670-672.
GLASS, C.K., HOLLOWAY, J.M., DEVARY, O.V. and ROSENFELD, M.G. (1988). The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response element. Cell 54, 313-323.
GODOWSKI, P.J. and KNIPE, D.M. (1983). Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. J. Virol. 47, 478-486.
GODOWSKI, P.J. and KNIPE, D.M. (1985). Identification of a herpes simplex virus function that represses late gene expression from parental viral genomes. J. Virol. 55, 357-365.
GODOWSKI, P.J., and KNIPE, D.M. (1986). Transcriptional control of herpesvirus gene expression: Gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA 83, 256-260.
GODOWSKI, P.J., RUSCONI, S., MIESFIELD, R. and YAMAMOTO, K.R. (1987). Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature 325, 365-368.
GOLDSTEIN, D.J. and WELLER, S.K. (1988a). Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. J. Virol. 62, 196-205.
GOLDSTEIN, D.J. and WELLER, S.K. (1988b). Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. Virlogy 166, 41-51.
GOLDSTEIN, D.J. and WELLER, S.K. (1988c). An ICP6::lacZ insertional mutagen to used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J. Virol. 62, 2970-2977.
GORMAN, C.M., MOFFATT, L.F., and HOWARD, B.H. (1982). Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol. Cell. Biol. 2, 1044-1051.
GRAHAM, F.L., VELDHEISEN, G. and WILKIE, N.M. (1973). Infectious herpesvirus DNA. Nature New Biology 245, 265-266.
GRAVES, B.J., JOHNSON, P.F., and McKNIGHT, S.L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell 44, 565-576.
GREEN, M., LOEWENSTEIN, P.M., PUSZTAI, R. and SYMINGTON, S.S. (1988). An adenovirus E1A protein domain activates

transcription in vivo and in vitro in the absence of protein synthesis. Cell 53, 921-926.
GREEN, M.R., TREISMAN, R., and MANIATIS, T. (1983). Transcriptional activation of cloned human beta-globin genes by viral immediate-early gene products. Cell 35, 137-148.
GREEN, S. and CHAMBON, P. (1987). Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. Nature 325, 75-78.
GREEN, S. and CHAMBON, P. (1988). Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics 4, 309-313.
GREEN, S., KUMAR, V., THEULAZ, I., WAHLI, W. and CHAMBON, P. (1988). The N-terminal DNA-binding "zinc-finger" of the oestrogen and glucocotricoid receptors determines gene specificity. EMBO J. 7, 3037-3044.
GUARENTE, L. (1988). UASs and enhancers: common mechanism of transcriptional activation in yeast and mammals. Cell 52, 303-305.
HAI, T., HORIKOSHI, M., ROEDER, R.G. and GREEN, M.R. (1988). Analysis of the the role of the transcription factor ATF in the assembly of a preinitiation complex. Cell 54, 1043-1051.
HALAZONETIS, T.D., GEORGOPOULOS, K., GREENBERG, M.E. and LEDER, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell 55, 917-924.
HALLIBURTON, I.W., RANDALL, R.E., KILLINGTON, R.A. and WATSON, D.H. (1977) Some properties of recombinants between type 1 and 2 herpes simplex viruses. J. Gen. Virol. 36, 471-484.
HARRIS-HAMILTON, E. and BACHENHEIMER, S.L.(1985). Accumulation of herpes simplex virus type 1 RNAs of different kinetic classes in the cytoplasm of infected cells. J. Virol. 53, 144-151.
HAY, R.T., and HAY, J. (1980). Properties of herpesvirus-induced "immediate early" polypeptides. Virology 104, 230-234.
HEARING, P. and SHENK, T. (1985). Sequence-independent autoregulation of the adenovirus type 5 Ela transcription unit. Mol. Cell. Biol. 5, 3214-3221.
HEINE, J.W., HONESS, R.W., CASSAI, E. and ROIZMAN, B. (1974). Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. 14, 640-651.
HEN, R., BORRELI, E. and CHAMBON, P. (1985). Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 Ela products. Science 230, 1391-1394.
HIGHLANDER, S.L., SUTHERLAND, S.L., GAGE, P.J., JOHNSON, D.C., LEVINE, M. and GLORIOSO, J.C. (1987). Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. 61, 3356-3364.
HILL, T.J. (1985). Herpes simplex virus latency, in: "The Herpesviruses" vol. 3, B.Roizman (ed.), Plenum Press, N.Y.
HILL, T.M., SINDEN, R.R. and SADLER, J.R. (1983). Herpes simplex virus types 1 and 2 induce shut-off of host protein synthesis by different mechanisms in Friend erythroleukaemia cells. J.Virol. 45, 241-250.
HILL, T.M., SADLER, J.R., BETZ, J.L. (1985). Virion component of herpes simplex virus type 1 KOS interferes withearly shut off of host protein synthesis induced by

herpes simplex virus type 2. J. Virol. 56, 312-316.
HOBSON, G.M., MITCHELL, M.T., MOLLOY, G.R., PEARSON, M.L. and BENFIELD, P.A. (1988). Identification of a novel TA-rich DNA binding protein that recognizes a TATA sequence within the brain creatinine kinase promoter. Nucleic Acids Res. 16, 8925-8944.
HOCHBERG, E. and BECKER, Y. (1968). Adsorption, penetration and uncoating of herpes simplex virus. J. Gen. Virol. 2, 231-241.
HOEFFLER, W.K., KOVELMAN, R. and ROEDER, R.G. (1988). Activation of transcription factor IIIC by the adenovirus Ela protein. Cell 53, 907-920.
HOLLAND, L.E., ANDERSON, K.P., SHIPMAN, C. and WAGNER, E.K. (1980). Viral DNA synthesis is required for the efficient expression of specific herpes simpex virus type 1 mRNA species. Virology 101, 10-24.
HOLLENBERG, S.M., GIGUERE, V., SEGUI, P. and EVANS, R.M. (1987). Colocalization of DNA-binding and transcriptional activation functiions in the human glucocorticoid receptor. Cell 49, 39-46.
HOLLENBERG, S.M. and EVANS, R.M. (1988). Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. Cell 55, 899-906.
HOLMES, A.M., WIETSTOCK, S.M. and RUYECHAN, W.T. (1988). Identification and characterization of a DNA primase activity present in herpes simplex virus type 1-infected cells. J. Virol. 62, 1038-1045.
HOLMES, S.M. and QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114, 193-197.
HOMA, F.L., OTAL, T.M., GLORIOSO, J.C., and LEVINE, M. (1986). Transcriptional control signals of a herpes simplex virus type 1 late ($gamma_2$) gene lie within bases -34 to +124 relative to the 5' terminus of the mRNA. Mol. Cell. Biol. 6, 3652-3666.
HONESS, R.W., and ROIZMAN, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14, 8-19.
HONESS, R.W., and ROIZMAN, B. (1975). Regulation of herpes virus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA 72, 1276-1280.
HOPE, I.A., MAHADEVAN, S. and STRUHL, K. (1988). Structural and functional characterization of the short acidic transcriptional activation region of yeast GCN4 protein. Nature 333, 635-640.
HOPE, I.A. and STRUHL, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell 46, 85-894.
HORIKOSHI, M., CAREY, M.F., KAKIDANI, H. and ROEDER, R.G. (1988a). Mechanism of action of a yeast activator: direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. Cell 54, 665-669.
HORIKOSHI, M., HAI, T., LIN, Y.-S., GREEN, M. and ROEDER, R.G. (1988b). Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell 54, 1033-1042.
HUBENTHAL-VOSS, J., HOUGHTEN, R.A., PEREIRA, L. and ROIZMAN, B. (1988). Mapping of functional and antigenic domains of the alpha 4 protein of herpes simplex virus 1. J. Virol. 62, 454-462.

HUBENTHAL-VOSS, J., STARR, L. and ROIZMAN, B. (1987). The herpes simplex virus origins of DNA synthesis in the S component are each contained in a transcribed open reading frame. J. Virol. 61, 3349-3355.
IKURA, K., BETZ, J.L., SADLER, J.R., and PIZER, L.I. (1983). RNAs transcribed from a 3.6-Kilobase SmaI fragment of the short unique region of the herpes simplex virus type 1 genome. J. Virol. 48, 460-471.
IMAGAWA, M., CHIU, R. and KARIN, M. (1987). Transcription factor AP-2 mediates induction by two different signal transduction pathways: protein kinase C and cAMP. Cell 51, 251-260.
IMPERIALE, M.J., FELDMAN, L.T., and NEVINS, J.R. (1983). Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis-acting adenovirus enhancer element. Cell 35, 127-136.
IMPERIALE, M.J., KAO, H.-T., FELDMAN, L.T., NEVINS, J.R. and STRICKLAND, S. (1984). Common control of the heat shock gene and early adenovirus genes: evidence for a cellular Ela-like activity. Mol. Cell. Biol. 4, 867-874.
JACKSON, S.P. and TJIAN, R. (1988). O-Glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. Cell 55, 125-133.
JACOB, R.J. and ROIZMAN, B. (1977). Anatomy of herpes simplex virus DNA. VII. Properties of the replicating DNA. J. Virol. 23, 294-411.
JACOB, R.J., MORSE, L.S. and ROIZMAN, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head to tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. 29, 448-457.
JAMIESON. A.T., GENTRY, G.A. and SUBAK-SHARPE, J.H. (1974). Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. 24, 465-480.
JAQUEMONT, B. and ROIZMAN, B. (1975). RNA synthesis in cells infected with herpes simplex virus. X. Properties of viral symmetric transcripts and of double stranded RNA prepared from them. J. Virol. 15, 707-713.
JAVIER, R.T., STEVENS, J.G., DISSETTE. V.B. and WAGNER, E.K. (1988). A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology 166, 254-257.
JOHNSON, D.C., and SPEAR, P.G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and egress of virions from infected cells. J. Virol. 43, 1102-1112.
JOHNSON, D.C., and SPEAR, P.G. (1984). Evidence for translational regulation of herpes simplex virus type 1 gD expression. J. Virol. 51, 389-394.
JOHNSON, D.C., WITTELS, M. and SPEAR, P.G. (1984). Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. J. Virol. 52, 238-247.
JOHNSON, D.C., and LIGAS, M.W. (1988). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. 62, 4605-4612.
JOHNSON, P.A. (1987). The control of herpes simplex virus late gene transcription. PhD Thesis, University of Glasgow.
JOHNSON , P.A., and EVERETT, R.D. (1986a). DNA replication

is required for abundant expression of a plasmid-borne late US11 gene of herpes simplex virus type 1. Nucleic Acids Res. 14, 3609-3625.
JOHNSON, P.A., and EVERETT, R.D. (1986b). The control of herpes simplex virus type 1 late gene transcription: A TATA-box/cap site region is sufficient for fully efficient regulated activity. Nucleic Acids Res. 14, 8247-8264.
JOHNSON, P.A., MacLEAN, C., MARSDEN, H.S., DALZIEL, R.G., and EVERETT, R.D. (1986). The product of gene US11 of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. 67, 871-883.
JONES, K.A. and TJIAN, R. (1985). Sp1 binds to promoter sequences and activates herpes simplex virus "immediate-early" gene transcription in vitro. Nature 317, 179-182.
JONES, K.A., YAMAMOTO, K.R., and TIJAN, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell 42, 559-572.
JONES, N.C., RIGBY, P.W.J. and ZIFF, E.B. (1988). Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes and Dev. 2, 267-281.
JONES, P.C., and ROIZMAN, B. (1979). Regulation of herpes virus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. 31, 299-314.
KADONAGA, J.T., CARNER, K.R., MASIARZ, F.R., and TJIAN, R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51, 1079-1090.
KADONAGA, J.T., JONES, K.A., and TIJAN, R. (1986). Promoter specific activation of RNA polymerase II transcription by Sp1. Trends in Biochemical Science 11, 20-23.
KAKIDANI, H. and PTASHNE, M. (1988). GAL4 activates gene expression in mammalian cells. Cell 52, 161-167.
KALDERON, D., ROBERTS, B.L., RICHARDSON, W.D., and SMITH, A.E. (1984). A short amino acid sequence able to specify nuclear location. Cell 39, 499-509.
KEEGAN, L., HILL, G. and PTASHNE, M. (1986). Separation of DNA binding from transcription-activating function of a eukaryotic regulatory protein. Science 231, 699-704.
KEIR, H., SUBAK-SHARPE, J.H., SHEDDEN, W.I.H., WATSON, D.H. and WILDY, P. (1966). Immunological evidence for a specific DNA polymerase produced after infection by herpes simplex virus. Virology 30, 154-157.
KEMP, L.M., PRESTON, C.M., PRESTON, V., and LATCHMAN, D.S. (1986). Cellular gene induction during herpes simplex virus infection can occur without viral protein synthesis. Nucleic Acids Res. 14, 9261-9270.
KENNEDY, P.G.E., LaTHANGUE, N.B., CHAN, W.L. and CLEMENTS, G.B. (1985). Cultured human neural cells accumulate a heat shock protein during acute herpes simplex infection. Neuroscience Letters, 61, 321-326.
KIEFF, E.D., BACHENHEIMER, S.L. and ROIZMAN, B. (1971). Size, composition and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. 8, 125-132.
KIEFF, E.D., HOYER, B., BACHENHEIMER, S.L. and ROIZMAN, B. (1972). Genetic relatedness of type and type 2 herpes simplex virus. J. Virol. 9, 738-745.
KIMELMAN, D., MILLER, J.S., PORTER, D. and ROBERTS, B.E.

(1985). Ela regions of the human adenoviruses and of the highly oncogenic simian adenovirus 7 are closely related. J. Virol. 53, 399-409.
KISHI, M., HARADA, H., TAKAHASHI, M., TANAKA, A., HAYASHI, M., NONOYAMA, M., JOSEPHS, S.F., BUCHNINDER, A., SCHACHTER, F., ABLASHI, D.V., WONG-STAHL, F., SALAHUDDIN, S.K. and GALLO, R.C. A repeat sequence, GGGTTA, is shared by DNA of human herpesvirus 6 and Mareks disease virus. J. Virol. 62, 4824-4827.
KIT, S. and DUBBS, D.R. (1963a). Acquisition of thymidine kinase activity by herpes simplex virus infected mouse fibroblast cells. Biochem. Biophys. Acta 11, 55-59.
KIT, S. and DUBBS, D.R. (1963b). Non-functional thymidine kinase cistron in bromodeoxyuridine resistant strains of herpes simplex virus. Biochem. Biophys. Res. Comm. 13, 500-504.
KIT, S., DUBBS, D.R., and SCHAFFER, P.A. (1978). Thymidine kinase activity in biochemically transformed mouse cells after superinfection by thymidine kinase-negative temperature-sensitive herpes simplex virus mutants. Virology 85, 456-463.
KNIGHT, G.B., GUDAS, J.M. and PARDEE, A.B. (1987) Cell-cycle-specific interaction of nuclear DNA-binding proteins with a CCAAT sequence from the human thymidine kinase gene. Proc. Natl. Acad. Sci. USA 84, 8350-8354.
KNIPE, D.M., BATTERSON, W., NOSAL, C., ROIZMAN, B., and BUCHAN, A. (1981). Molecular genetics of herpes simplex virus. VI. Characterisation of a temperature sensitive mutant defective in the expression of all early viral gene products. J. Virol. 38, 539-547.
KNIPE, D.M., and SMITH, J.L. (1986). A mutant herpesvirus protein leads to a block in nuclear localisation of other viral proteins. Mol. Cell. Biol. 6, 2371-2381.
KNIPE, D.M., SENECHEK, D., RICE, S.A. and SMITH, J.L. (1987). Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. J. Virol. 61, 276-284.
KNOPF, K.W. (1979). Properties of herpes simplex virus DNA polymerase and characterization of its associated activity. European Journal of Biochemistry 98, 231-244.
KO, H.-S., FAST, P., McBRIDE, W. and STAUDT, L.M. (1988). A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. Cell 55, 135-144.
KOFF, A. and TEGTMEYER, P. (1988) Characterization of major recognition sequences for a herpes simplex virus type 1 origin-binding protein. J. Virol. 62, 4096-4103.
KOVESDI, I., REICHEL, R. and NEVINS, J.R. (1986). Identification of a cellular transcription factor involved in E1A trans-activation. Cell 45, 219-228.
KOZAK, M. and ROIZMAN, B. (1974). Regulation of herpesvirus macromolecular synthesis: nuclear retention of non-translated viral RNA sequences. Proc. Natl. Acad. Sci. USA 71, 4322-4326.
KRIPPL, B., FERGUSON, B., JONES, N., ROSENBERG, M. and WESTPHAL, H. (1985). Mapping of functional domains in adenovirus Ela proteins. Proc. Natl. Acad. Sci. USA 82, 7480-7484.
KRISTIE, T.M., and ROIZMAN, B. (1984). Separation of sequences defining basal expression from those conferring alpha gene recognition within the regulatory domains of herpes simplex virus 1 alpha genes. Proc. Natl. Acad. Sci.

USA 81, 4065-4069.
KRISTIE, T.M., and ROIZMAN, B. (1986a). Alpha 4, the major regulatory protein of herpes simplex type 1, is stably and specifically associated with promoter-regulatory domains of alpha genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA 83, 3218-3222.
KRISTIE, T.M., and ROIZMAN, B. (1986b). DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 83, 4700-4704.
KRISTIE, T.M. and ROIZMAN, B. (1987). Host cell proteins bind to the cis-acting site required for virion mediated induction of herpes simplex virus 1 alpha genes. Proc. Natl. Acad. Sci. USA 84, 71-74.
KUMAR, V. and CHAMBON, P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced monodimer. Cell 55, 145-156.
KUMAR, V., GREEN, S., STACK, G., BERRY, M., JIN, J.-R. and CHAMBON, P. (1987). Functional domains of the human estrogen receptor. Cell 51, 941-951.
KWONG, A.D. and FRENKEL, N. (1987). Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc. Natl. Acad. Sci. USA 84, 1926-1930.
KWONG, A.D., KRUPER, J.A. and FRENKEL, N. (1988). Herpes simplex virus virion host shutoff function. J. Virol. 912-921.
LADIN, B.F., BLANKENSHIP, M.L. and BEN-PORAT, T. (1980). Replication of herpes virus DNA. V. Maturation of concatemeric DNA of pseudorabies virus to genome length is related to capsid formation. J. Virol. 33, 1151-1164.
LANG, J.C., SPANDIDOS, D.A., and WILKIE, N.M. (1984). Transcriptional regulation of a herpes simplex virus immediate early gene is mediated through an enhancer-type sequence. EMBO J. 3, 389-395.
LANGELAND, N., HOLMSEN, H., LILLEHAUG, J.R and HAARR, L. (1987). Evidence that neomycin inhibits binding of herpes simplex virus type 1 to the cellular receptor. J. Virol. 61, 3388-3393.
LANGER, S.J. and OSTROWSKI, M.C. (1988). Negative regulation of transcription in vitro by a glucocorticoid response element is mediated by a trans-acting factor. Mol. Cell. Biol. 8, 3872-3881.
LATCHMAN, D.S. (1988). Effect of herpes simplex virus type 2 infection on mitochondrial gene expression. J. Gen. Virol. 69, 1405-1410.
LATCHMAN, D.S., ESTRIDGE, J.K. and KEMP, L.M. (1987). Transcriptional induction of the ubiquitin gene during herpes simplex virus infection is dependent upon the viral immediate-early protein ICP4. Nucleic Acids Res. 15, 7283-7293.
LaTHANGUE, N.B., SHRIVER, K., DAWSON, C., and CHAN, W.L. (1984). Herpes simplex virus infection causes the accumulation of a heat shock protein. EMBO J. 3, 267-277.
LECH, K., ANDERSON, K. and BRENT, R. (1988). DNA-bound Fos proteins activate transcription in yeast. Cell 52, 179-184.
LEE, W., HASLINGER, A., KARIN, M. and TJIAN, R. (1987a). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene. Nature 325, 368-372.

LEE, W., MITCHELL, P. and Tjian, R. (1987b). Purified transcription factor Ap-1 interacts with TPA-inducible enhancer elements. Cell 49, 741-752.
LEIDEN, J.M., BUTTYAN, R., and SPEAR, P.G. (1976). Herpes simplex virus expression in transformed cells. I. Regulation of the viral thymidine kinase gene in transformed L cells by products of superinfecting virus. J. Virol. 20, 413-424.
LEINBECH, S.S., RENO, J.M., LEE, L.F., ISBELL, A.F. and BOEZI, J.A. (1976). Mechanism of phosphonoacetate inhibition of herpes-induced DNA polymerase. Biochemistry 15, 426-430.
LEONG, K., BRUNET, L. and BERK, A.J. (1988). Factors responsible for the higher transcriptional activity of extracts of adenovirus-infected cells fractionate with the TATA-box transcription factor. Mol. Cell. Biol. 8, 1765-1774.
LEVINE, M. and HOEY, T. (1988). Homeobox proteins as sequence-specific transcription factors. Cell 55, 537-540.
LIGAS, M.W. and JOHNSON, D.C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. 62, 1486-1494.
LILLIE, J.W., GREEN, M. and GREEN, M.R. (1986). An adenovirus Ela protein region required for transformation and transcriptional repression. Cell 46, 1043-1051.
LILLIE, J.W., LOEWENSTEIN, P.M., GREEN, M.R. and GREEN, M. (1987). Functional domains of adenovirus Ela proteins. Cell 50, 1091-1100.
LIN, Y.-S., CAREY, M.F., PTASHNE, M. and GREEN, M.R. (1988). GAL4 derivatives function alone and synergistically with mammalian activators in vitro. Cell 54, 659-664.
LOCKSHON, D. and GALLOWAY, D.A. (1988). Sequence and structural requirements of a herpes simplex viral DNA replication origin. Mol. Cell. Biol. 8, 4018-4027.
LONGNECKER, R., and ROIZMAN, B. (1986). Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the alpha 47 gene. J. Virol. 58, 583-591.
LONGNECKER, R., and ROIZMAN, B. (1987). Clustering of genes dispensable for growth in culture in the S components of the HSV-1 genome. Science 236, 573-576.
LONSDALE, D.M. (1979). A rapid technique for distinguishing herpes simplex virus type 1 from type 2 by restriction technology. Lancet i, 849-852.
MacLEAN, A.R. and BROWN, S.M. (1987). A Herpes simplex virus type 1 variant which fails to synthesize immediate early polypeptide Vmw63. J. Gen. Virol. 68, 1339-1350.
MacPHERSON, I. and STOKER, M. (1962). Polyoma transformation of hamster cell clones-an investigation of genetic factors affecting cell competence. Virology 16, 147-151.
McCARTHY, A.M., McMAHAN, L. and SCHAFFER, P.A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. 63, 18-27.
McGEOCH, D.J., DALRYMPLE, M.A., DAVISON, A.J., DOLAN, A., FRAME, M.C., McNAB, D., PERRY, L.J., SCOTT, J.E. and TAYLOR, P. (1988b). The complete nucleotide sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69, 1531-1574.

McGEOCH, D.J., DALRYMPLE, M.A., DOLAN, A., McNAB, D., PERRY, L.J., TAYLOR, P. and CHALLBERG, M.D. (1988a). Structures of herpes simplex virus type 1 genes required for replication of virus DNA. J. Virol. 62, 444-453.

McGEOCH, D.J., DOLAN, A., DONALD, S., and RIXON, F.J. (1985). Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. 181, 1-13.

McGEOCH, D.J., DOLAN, A., DONALD, S., and BRAUER, D.H.K. (1986). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Res. 14, 1727-1764.

McGEOCH, D.J., MOSS, H.W.M., McNAB, D. and FRAME, M.C. (1987). DNA sequence and genetic content of the HindIII 1 region in the short unique component of the herpes simplex virus type 2 genome: identification of the gene encoding glycoprotien G, and evolutionary comparisons. J. Gen. Virol. 68, 19-38.

McKNIGHT, J.L.C., PELLET, P.E., JENKINS, F.J. and ROIZMAN, B. (1987). Characterization and nucleotide sequence of two herpes simplex virus 1 genes whose products modulate alpha-trans-inducing factor dependant activation of alpha genes. J. Virol. 61, 992-1001.

McKNIGHT, S., and TIJAN, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. Cell 46, 795-805.

McKNIGHT, S., KINGSBURY, R., EISENBERG, S., TUFARO, F., WEINHEIMER, S., TRIEZENBERG, S., JOHNSON, P. and GRAVES, B. (1985). Studies on the promoter of the herpes simplex thymidine kinase gene, in: "Eukaryotic Transcription" ppl16-122, Y.Gluzman (ed.), Cold Spring Harbor Lab., N.Y.

MA, J. and PTASHNE, M. (1987a). Deletion analysis of GAL4 defines two transcriptional activating segments. Cell 48, 847-853.

MA, J. and PTASHNE, M. (1987b). The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80. Cell 50, 137-142.

MA, J. and PTASHNE, M. (1987c). A new class of yeast transcription activators. Cell 51, 113-119.

MA, J. and PTASHNE, M. (1988). Converting a eukaryotic transcriptional inhibitor into an activator. Cell 55, 443-446.

MACKEM, S., and ROIZMAN, B. (1982a). Differentiation between alpha promoter and regulator regions of herpes simplex virus 1: The functional domains and sequence of a movable alpha regulator. Proc. Natl. Acad. Sci. USA 79, 4917-4921.

MACKEM, S., and ROIZMAN, B. (1982b). Structural features of the herpes simplex virus alpha gene 4, 0 and 27 promoter-regulatory sequences which confer alpha regulation on chimeric thymidine kinase genes. J. Virol. 44, 939-949.

MACNAB, J.C.M. (1987). Herpes simplex virus and human cytomegalovirus: their role in morphological transformation and genital cancers. J. Gen. Virol. 68, 2525-2550.

MACNAB, J.C.M., ORR, A., and LA THANGUE, N.B. (1985). Cellular proteins expressed in herpes simplex virus transformed cells also accumulate on herpes simplex virus infection. EMBO J. 4, 3223-3228.

MANIATIS. T., GOODBURN, S. and FISCHER, J.A. (1987). Regulation of inducible and tissue-specific gene expression. Science 236, 1237-1245.

MARCHETTI, M.E., SMITH, C.A. and SCHAFFER, P.A. (1988). A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in U_L. J. Virol. 62, 715-721.

MARSDEN, H.S., CAMPBELL, M.E.M., HAARR, L., FRAME, M.C., PARRIS D.S., MURPHY, M., HOPE, R.G., MULLER, M.T. and PRESTON, C.M. (1987). The 65,000-M_r DNA binding and trans-inducing proteins of herpes simplex virus type 1. J. Virol. 61, 2428-2437.

MARSDEN, H.S., CROMBIE, I.K., and SUBAK-SHARPE, J.H. (1976). Control of protein synthesis in herpesvirus-infected cells: analysis of the polypeptides induced by wild type and sixteen temperature-sensitive mutants of HSV strain 17. J. Gen. Virol. 31, 347-372.

MARSDEN, H.S., LANG, J., DAVISON, A.J., HOPE, R.G., and MacDONALD, D.M. (1982). Genomic location and lack of phosphorylation of the HSV immediate early-polypeptide IE12. J. Gen. Virol. 62, 17-27.

MARSDEN, H.S., STOW, N.D., PRESTON, V.G., TIMBURY, M.C. and WILKIE, N.M. (1978). Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. 28, 624-642.

MARX, J.L. (1988) Homeobox linked to gene control. Science 242, 1008-1009.

MATTHEWS, R.E.F. (1982). Classification and nomenclature of viruses. Fourth report of the International Comittee on Taxonomy of Viruses. Intervirology 17, 1-199.

MATSUI, T., SEGAIL, J., WEIL, P.A. and ROEDER, R.G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. J. Biol. Chem. 255, 11992-11996.

MAVROMARA-NAZOS, P. and ROIZMAN, B. (1987). Activation of herpes simplex virus 1 $gamma_2$ genes by viral DNA replication. Virology, 161, 593-598.

MAVROMARA-NAZOS, P., SILVER, S., HUBENTHAL-MOSS, J., McKNIGHT, J.L.C., and ROIZMAN, B. (1986). Regulation of herpes simplex virus genes: alpha gene sequence requirements for transient induction of indicator genes regulated by beta or late ($gamma_2$) promoters. Virology 149, 152-164.

MAXAM, A.M., and GILBERT, W. (1977). A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA 74, 560-564.

MAXAM, A.M., and GILBERT, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Methods in Enzymology 65, 499-560.

MEIGNIER, B., LONGNECKER, R., MAVROMARA-NAZOS, P., SEARS, A.E. and ROIZMAN, B. (1988). Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus type 1. Virology 162, 251-254.

MELLERICK, D.M. and FRASER, N.W. (1987). Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology 158, 265-275.

METZLER, D.W., and WILCOX, K.W. (1985). Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. 55, 329-337.

MICHAEL, N., SPECTOR, D., MAVROMARA-NAZOS, P., KRISTIE, T.M., and ROIZMAN, B. (1988). The DNA-binding properties of the major regulatory protein alpha 4 of herpes simplex virus. Science 239, 1531-1534.

MITCHELL, P.J., WANG, C. and TJIAN, R. (1987). Positive and

negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell 50, 847-861.
MITTNACHT, S., STRAUB, P., KIRCHNER, H. and JACOBSEN, H. (1988). Interferon treatment inhibits onset of herpes simplex virus immediate-early transcription. Virology 164, 201-210.
MONCOLLIN, V., MIYAMOTO, N.G., ZHENG, X.M., and EGLY, J.M. (1986). Purification of a factor specific for the upstream element of the adenovirus-2 major late promoter. EMBO J. 5, 2577-2584.
MORAN, E. (1988). A region of SV40 large T antigen can substitute for a transforming domain of the adenovirus E1a products. Nature 334, 168-170.
MORAN, E., and MATHEWS, M.B. (1987). Multiple functional domains in the adenovirus E1A gene. Cell 48, 177-178.
MORAN, E. ZERLER, B., HARRISON, T.M., and MATHEWS, M.B. (1986). Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. Mol. Cell Biol. 6, 3470-3480.
MORSE, B.L., BUCHMAN, T.G., ROIZMAN, B. and SCHAFFER, P.A. (1977). Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 x HSV-2) recombinants. J. Virol. 24, 231-248.
MOSCA, J.D., REYES, G.R., PITHA, P.M., and HAYWARD, G.S. (1985). Differential activation of hybrid genes containing immediate-early or delayed-early promoters after superinfection of stable DNA-transfected cell lines. J. Virol. 56, 867-878.
MOSS, H. (1986). The herpes simplex virus type 2 alkaline DNase activity is essential for replication and growth. J. Gen. Virol. 67, 1173-1178
MULLER, M.T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. 61, 858-865.
MULLER, M.T., BOLLES, L.S. and PARRIS, D.S. (1985). Association of type 1 DNA topoisomerase with herpes simplex virus. J. Gen. Virol. 66, 1565-1574.
MUNK, K. and SAUER, G. (1964). Relationship between cell DNA metabolism and nucleoplasmic alterations in herpes virus infected cells. Virology 22, 153-154.
MUNOZ, A., ZENKE, M., GEHRING, J., SAP, J., BEUG, H. and VENNSTROM, B. (1988). Characterization of the hormone-binding domain of the chicken c-erbA/thyroid hormone receptor protein. EMBO J. 7, 155-159.
MURCHIE, M.-J. and McGEOCH, D.J. (1982) DNA sequence analaysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950 to 0.978). J. Gen. Virol. 62, 1-15.
NAKABEPPU, Y., RYDER, K. and NATHANS, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell 55, 907-915.
NAKAJIMA, N., HORIKOSHI, M. and ROEDER, R.G. (1988). Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity and TATA box-promoter interactions of TFIID. Mol. Cell. Biol. 8, 4028-4040.
NALBANTOGLU, J., PHEAR, G.A., and MEUTH, M. (1986). Nucleotide sequence of hamster adenine phosphoribosyl transferase (aprt) gene. Nucleic Acids Res. 14, 1914.

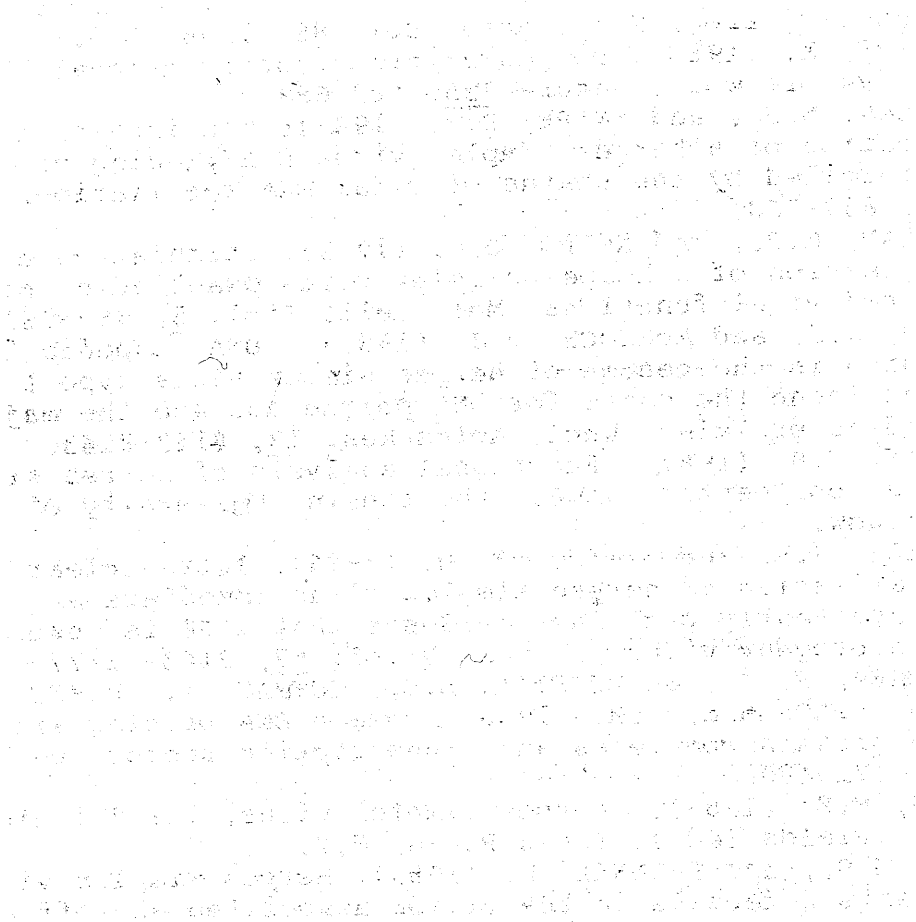
NISHIOKA, Y., and SILVERSTEIN, S. (1977). Degradation of cellular mRNA during infection by herpes simplex virus. Proc. Natl. Acad. Sci. USA 74, 2370-2374.
NISHIOKA, Y., and SILVERSTEIN, S. (1978). Requirement of protein synthesis for the degradation of host mRNA in Friend erythroleukaemia cells infected with herpes simplex virus type 1. J. Virol. 27, 619-627.
NOMIYAMA, H., FROMENTAL, C., XIAO, J.H. and CHAMBON, P. (1987). Cell-specific activity of the constituent elements of the simian virus 40 enhancer. Proc. Natl. Acad. Sci. USA 84, 7881-7885.
NONET, M., SWEETSER, D. and YOUNG, R.A. (1987). Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. Cell 50, 909-915.
NOTARIANNI, E.L., and PRESTON, C.M. (1982). Activation of cellular stress protein genes by herpes simplex virus temperature-sensitive mutants which overproduce immediate early polypeptides. Virology 123, 113-122.
OBERMAN, F. and PANET, A. (1988). Inhibition of transcription of herpes simplex virus immediate early genes in interferon-treated human cells. J. Gen. Virol. 69, 1167-1177.
O'HARE, P., and HAYWARD, G.S. (1984). Expression of recombinant genes containing herpes simplex virus delayed-early regulatory regions and trans activation by herpes virus infection. J. Virol. 52, 522-531.
O'HARE, P., and HAYWARD, G.S. (1985a). Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. 53, 751-760.
O'HARE, P., and HAYWARD, G.S. (1985b). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. 56, 723-733.
O'HARE, P., and HAYWARD, G.S. (1987). Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded trans-acting factors. J. Virol. 61, 190-199.
O'HARE, P. and GODING, C.R. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell 52, 435-445.
OLIVO, P.D., NELSON, N.J. and CHALLBERG, M.D. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. Proc. Natl. Acad. Sci. USA 85, 5414-5418.
OROSKAR, A.A. and READ, G.S. (1987). A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. J. Virol. 61, 604-606.
PALFREYMAN, J.W., MacLEAN, J.B., MESSEDER, E., and SHEPPARD, R.C. (1984). Successful use of oligo peptides as immunogens in the preparation of antisera to immediate-early gene products of herpes simplex virus type 1. J. Gen. Virol. 65, 865-874.
PARA, M.F., BAUCHE, R.B. and SPEAR, P.G. (1980). Immunoglobulin G(Fc)-binding receptors on virions of herpes simplex type 1 and transfer of these receptors to the cell surface by infection. J. Virol. 34, 512-520.
PARKER, C.S. and TOPOL, J. (1984). A *Drosophila* RNA polymerase II transcription factor binds to the regulatory

site of an hsp70 gene. Cell 37, 273-283.
PARRIS, D.D., CROSS, A., HAARR, L., ORR, A., FRAME, M.C., MURPHY, M., McGEOCH, D.J. and MARSDEN, H.S. (1988). Identification of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. J. Virol. 62, 818-825.
PARSLOW, T.G., JONES,S.D., BOMD, B. and YAMAMOTO, K.R. (1987). The immunoglobulin octanucleotide: independent activity and selective interaction with enhancers. Science 235, 1498-1501.
PATEL, R., CHAN, W.L., KEMP, L.M., LaTHANGUE, N.B., and LATCHMAN, D.S. (1986). Isolation of cDNA clones derived from a cellular gene transcriptionally induced by herpes simplex virus. Nucleic Acids Res. 14, 5629-5640.
PATERSON, T. and EVERETT, R.D. (1988a) Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. Virology 166, 186-196.
PATERSON, T. and EVERETT, R.D. (1988b). The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation. Nucleic Acids Res. 16, 11005-11023
PEAT, D.S. and STANLEY, M.A. (1986). Chromosome damage induced by herpes simplex virus type 1 in early infection. J. Gen. Virol. 67, 2273-2277.
PEDERSEN, M., TALLEY-BROWN, S. and MILLET, R.C. (1981). Gene expression of herpes simplex virus. III. Effect of arabinosyladenine on viral polypeptide synthesis. J. Virol. 38, 712-719.
PELLET, P.E., McKNIGHT, J.L.C., JENKINS, F.J. and ROIZMAN, B. (1985). Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing alpha genes. Proc. Natl. Acad. Sci., USA 82, 5870-5874.
PERDUE, M.L., COHEN, J.C., RANDALL, C.C. and O'CALLAGHAN, D.J. (1976). Biochemical studies of the maturation of herpesvirus nucleocapsid species. Virology 74, 194-208.
PEREIRA, L., WOLFF, M.H., FENWICK, M., and ROIZMAN, B. (1977). Regulation of herpesvirus macromolecular synthesis. V. Properties of polypeptides made in HSV-1 and HSV-2 infected cells. Virology 77, 733-749.
PERRY, L.J., RIXON, F.J., EVERETT, R.D., FRAME, M.C., and McGEOCH, D.J. (1986). The IE110 gene of herpes simplex virus type 1: characterisation by mRNA mapping, DNA sequence, oligopeptide antiserum and mutational analysis. J. Gen. Virol. 67, 2365-2380.
PERSSON, R.H., BACCHETTI, S., and SMILEY, J.R. (1985). Cells that constitutively express the herpes simplex virus immediate-early protein ICP4 allow efficient activation of viral delayed-early genes in trans. J. Virol. 54, 414-421.
PERSSON, R.H. and BACCHETTI, S. (1987). In cell lines constitutvely synthesizing a temperature-sensitive ICP4 protein of herpes simplex virus type 1, amount and function of ICP4 are both regulated by temperature. Virology 158, 285-293.
PICARD, D., SALSER, S.J. and YAMAMOTO, K.R. (1988). A movable and regulable inactivation function withion the steroid binding domain of the glucocorticoid receptor. Cell 54, 1073-1080.
PICARD, D. and YAMAMOTO, K.R. (1987). Two signals mediate hormone-dependent nuclear localization of the

glucocorticoid receptor. EMBO J. 6, 3333-3340.
PILON, L., LANGELIER, Y. and ROYAL, A. (1986). Herpes simplex virus type 2 mutagenesis: characterization of mutants induced at the hprt locus of non-permissive XC cels. Mol. Cell. Biol. 6, 2977-2983.
PILON, L., ROYAL, A. and LANGELIER, Y. (1985). Increased mutation frequency after herpes simplex type 2 infection of non-permissive XC cells. J. Gen. Virol. 66, 259-265.
PIZER, L.I. and BEARD, P. (1976). The effect of herpes virus infection on mRNA in polyoma virus transformed cells. Virology 75, 477-480.
PIZER, L.I., TEDDER, D.G., BETZ, J.L., WILCOX, K.W., and BEARD, P. (1986). Regulation of transcription in vitro from herpes simplex virus genes. J. Virol. 60, 950-959.
POLVINO-BODNAR, M., ORBERG, P.K. and SCHAFFER, P.A. (1987). Herpes virus type 1 ori_L is not required for viral replication or for the establishment and reactivation of latent infection in mice. J. Virol. 61, 3528-3535.
POST, L.E., MACKEM, S., and ROIZMAN, B. (1981). Regulation of alpha genes of herpes simplex virus; expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. Cell 24, 555-565.
POST, L.E., and ROIZMAN, B. (1981). A generalised technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. Cell 25, 227-232.
POWELL, K.L., LITTLER, E. and PURIFOY, D.J.M. (1981). Non-structural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J. Virol. 39, 894-902.
POWELL, K.L., PURIFOY, D.J.M. and COURTNEY, R.J. (1975). The synthesis of herpes simplex virus proteins in the absence of DNA synthesis. Biochem. Biophys. Res. Comm. 66, 262-271.
PRESTON, C.M. (1979a). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. J. Virol. 29, 275-284.
PRESTON, C.M. (1979b). Abnormal properties of an immediate early polypeptide in cells infected with the herpes simplex virus type 1 mutant tsK. J. Virol. 32, 357-369.
PRESTON, C.M. and CORDINGLEY, M.G. (1982). mRNA- and DNA-directed synthesis of herpes simplex virus-coded exonuclease in Xenopus laevis oocytes. J. Virol. 43, 386-394.
PRESTON, C.M., CORDINGLEY, M.G., and STOW, N.D. (1984). Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate early gene. J. Virol. 50, 708-716.
PRESTON, C.M., FRAME, M.C. and CAMPBELL, M.E.M. (1988). A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell 52, 425-434.
PRESTON, C.M., and NOTARIANNI, E.L. (1983). Poly(ADP-ribosyl)ation of a herpes simplex virus immediate-early polypeptide. Virology 131, 492-501.
PRESTON, C.M., and TANNAHILL, D. (1984). Effects of orientation and position on the activity of a herpes simplex virus immediate early gene far-upstream region. Virology 137, 439-444.
PRESTON, V.G. (1981). Fine-structure mapping of herpes simplex virus type 1 temperature-sensitive mutations

within the short repeat region of the genome. J. Virol. 39, 150-161.
PRESTON V.G. and FISHER, F.B. (1984). Identification of the herpes simplex virus type 1 gene encoding the dUTPase. Virology 138, 58-68.
PRESTON, V.G., PALFREYMAN, J.W. and DUTIA, B.M. (1984). Identification of a herpes simplex virus type I polypeptide which is a component of the virus induced ribonucleotide reductase. J. Gen. Virol. 65, 1457-1466.
PURIFOY, D.J.M., LEWIS, R.B. and POWELL, K.L. (1977). Identification of the herpes simplex virus DNA polymerase gene. Nature 269, 621-623.
PRIVALSKY, M.L., BOUCHER, P., KONING, A. and JUDELSON, C. (1988). Genetic dissection of functional domains within the avian erythroblastosis virus v-erbA oncogene. Mol. Cell. Biol. 8, 4510-4517.
PRUIJN, G.J.M., VAN DRIEL, W. and VAN DER VLIET (1986). Nuclear factor III, a novel sequence-specific DNA binding protein from HeLa cells stimulating adenovirus DNA replication. Nature 322, 656-659.
PRYWES, R., DUTA, A., CROMLISH, J.A. and ROEDER, R.G. (1988). Phosphorylation of serum response factor, a factor that binds to the serum response element of the c-fos enhancer. Proc. Natl. Acad. Sci. 85, 7206-7210.
PTASHNE, M. (1988). How eukaryotic transcriptional activators work. Nature 335, 683-689.
QUINLAN, M.P., and KNIPE, D.M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell 36, 857-868.
QUINLAN, M.P., and KNIPE, D.M. (1985). Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. 5, 957-963.
QUINN, J.P. and McGEOCH, D.J. (1985). DNA sequence of the region in the genome of herpes simpex virus type 1 containing the genes for DNA polymerase and the major DNA binding protein. Nucl. Acids Res. 13, 8143-8163.
RAMSAY, F.H. (1987). Functional analysis of herpes simplex virus polypeptide Vmw65. PhD thesis, University of Glasgow.
RANDALL, R.E. and DINWOODIE, N. (1986). Intranuclear localization of herpes simplex virus immediate-early and delayed-early proteins: evidence that ICP4 is associated with progeny virus. J. Gen. Virol. 67, 2163- 2177.
RAUSCHER, F..J., SAMBUCETTI. L.C., CURRAN, T., DISTEL, R.J. and SPIEGELMAN, B.M. (1988). Common DNA binding site for FOS protein complexes and transcription factor Ap-1. Cell 52, 471-480.
RAWLS, W.E. (1985). Herpes simplex virus, in: "Virology", B.N. Fields (ed.), Raven Press, N.Y.
READ, G.S., and FRENKEL, N. (1983). Herpes simplex virus mutants defective in the virion associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate-early) viral polypeptides. J. Virol. 46, 498-512.
REYES, G.R., LaFEMINA, R., HAYWARD, S.D. and HAYWARD, G.S. (1979). Morphological transformation by DNA fragments of human herpesviruses: evidence for two distinct transforming regions in HSV-1 and HSV-2 and lack of correlation with biochemical transfer of the thymidine kinase gene. Cold Spring Harbor Symposia on Quantitative Biology 44, 629-641.

RIGBY, P.W.J., DIECKMANN, M., RHODES, C. AND BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237-251.



RICE, S.A. and KNIPE, D.M. (1988). Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. J. Virol. 62, 3814-3823.
RICHTER, J.D., SLAVICEK, J.M., SCHNEIDER, J.F. and JONES, N.C. (1988). Heterogeneity of adenovirus type 5 E1a proteins: multiple serine phosphorylations induce slow migrating electrophoretic variants but do not affect E1a-induced transcriptional activation or repression. J. Virol. 62, 1948-1955.
RIXON, F.J., CAMPBELL, M.E., and CLEMENTS, J.B. (1982). The immediate-early mRNA that encodes the regulatory polypeptide Vmw175 of herpes simplex virus type 1 is unspliced. EMBO J. 1, 1273-1277.
ROBERTS, M.S., BOUNDY, A., O'HARE, P., PIZZORNO, M.C., CIUFO, D.M. and HAYWARD, G.S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. 62, 4307-4320.
ROBERTSON, M. (1988). Homoeo boxes, POU proteins and the limits to promiscuity. Nature 336, 522-524.
ROCK, D.L. and FRASER, N.W. (1983). Detection of HSV-1 genome in central nervous system of latently infected mice. Nature 302, 523-525.
ROCK, D.L. and FRASER, N.W. (1985). Latent herpes simplex virus type 1 DNA contains 2 copies of the virion DNA joint region. J. Virol. 55, 849-852.
ROCK, D.L., NESBURN, A.B., GHIASI, H., ONG, J., LEWIS, T.L., LOHENSGARD, J.R. and WESCHLER, S.L. (1987). Detection of latency-related transcripts in trigeminal ganglia of rabbits infected with herpes simplex virus type 1. J. Virol. 61, 3820-3826.
ROIZMAN, B. (1969). The herpesviruses- a biochemical definition of the group. Current Topics in Microbiology and Immunology 49, 3-79.
ROIZMAN, B. (1982). The family herpesviridae: general description, taxonomy and classification, in: "The Herpesviruses" vol.I, ppl-23, B. Roizman (ed.), Plenum Press, N.Y.
ROIZMAN, B. and BATTERSON, W. (1985). Herpesviruses and their replication, in: "Virology" pp497-526, B.N.Fields (ed.), Raven Press, N.Y.
ROIZMAN, B. and FURLONG, D. (1974). The replication of herpesviruses, in: "Comprehensive Virology" vol.3, pp229-403, H. Fraenkel-Conrat and R.R. Wagner (eds.), Plenum Press, N.Y.
ROIZMAN, B. and ROANE, P.R. (1964). The multiplication of herpes simplex virus. II. The relationship between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology, 22, 262-269.
ROSALES, R., VIGNERON, M., MACCHI, M., DAVIDSON, I., XIAO, J.H. and CHAMBON, P. (1987). *In vitro* binding of cell-specific and ubiquitous nuclear proteins to the octamer motif of the SV40 enhancer and related motifs present in other promoters and enhancers. EMBO J. 6, 3015-3025.
ROSENTHAL, K.S., LEUTHER, M.D. and BARISAS, B.G. (1984). Herpes simplex virus binding and entry modulate cell surface protein mobility. J. Virol. 49, 980-983.
RUSSELL, J., STOW, E.C., STOW, N.D., and PRESTON, C.M. (1987a). Abnormal forms of the herpes simplex virus immediate early polypeptide Vmw175 induce the cellular

stress response. J. Gen. Virol. 68, 2397-2406.
RUSSELL, J., STOW, N.D., STOW, E.C., and PRESTON, C.M. (1987b). Herpes simplex virus genes involved in latency in vitro. J. Gen. Virol. 68, 3009-3018.
RUSSELL, J. and PRESTON, C.M. (1986). An in vitro latency system for herpes simplex virus type 2. J. Gen. Virol. 67, 397-403.
SACKS, W.R., GREENE, C.C., ASCHMAN, D.P., and SCHAFFER, P.A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. 55, 796-805.
SACKS, W.R., and SCHAFFER, P.A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. 61, 829-839.
SADOWSKI, I., MA, J., TRIEZENBERG, S. and PTASHNE, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. Nature 335, 563-564.
SALAHUDDIN, S.Z., ALBASWHI, D.V., MARKEM, P.D., JOSEPHS, S.F., STURZENEGGER, S., KAPLAN, M., HALLIGAN, G., BIBENFIELD, P., WONG-STAHL, F., KRAMANSKY, B. and GALLO, R.C. (1986). Isolation of a new virus HBLV, in patients with lymphoproliferative disorders. Science 234, 596-601.
SANDRI-GOLDIN, R.M., GOLDIN, A.L., HOLLAND, L.E., GLORIOSO, J.C., and LEVINE, M. (1983). Expression of herpes simplex virus beta and gamma genes integrated in mammalian cells and their induction by an alpha gene product. Mol. Cell. Biol. 3, 2028-2044.
SANDRI-GOLDIN, R.M., SEKULOVICH, R.E., and LEARY, K. (1987). The alpha protein ICP0 does not appear to play a major role in the regulation of herpes simplex virus gene expression during infection in tissue culture. Nucleic Acids Res. 15, 905-919.
SAP, J., MUNOZ, A., DAMM, K., GOLDBERG, Y., GHYSDAEL, J., LEUTZ, A., BEUG, H. and VENNSTROM, B. (1986). The c-erbA protein is a high affinity receptor for thyroid hormone. Nature 324, 635-640.
SASSONE-CORSI, P. (1988). Cyclic AMP induction of early adenovirus promoters involves sequences required for Ela trans-activation. Proc. Natl. Acad. Sci. USA 85, 7192-7196.
SASSONE-CORSI, P., LAMPH, W.W., KAMPS, M. and VERMA, I. (1988). fos-associated cellular protein p39 is related to nuclear transcription factor Ap-1. Cell 54, 553-560.
SAWADOGO, M., and ROEDER, R.G. (1985a). Factors involved in specific transcription by human RNA polymerase II: Analysis by a rapid and quantitative in vitro assay. Proc. Natl. Acad. Sci. USA 82, 4394-4398.
SAWADOGO, M., and ROEDER, R.G. (1985a). Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box. Cell 43, 165-175.
SCHEIDEREIT, C., HEGUY, A. and ROEDER, R.G. (1987). Identification and purification of a human lymphoid-specific octamer-binding protein (OTF-2) that activates transcription of an immunoglobulin promoter in vitro. Cell 51, 783-793.
SCHEK, N. and BACHENHEIMER, S.L. (1985). Degradation of cellular mRNAs by a virion-associated factor during herpes simplex virus infection of Vero cells. J. Virol. 55, 601-610.
SCHNEIDER, J.F., FISHER, F., GODING, C.R., and JONES, N.C. (1987). Mutational analysis of the adenovirus Ela gene:

the role of transcriptional regulation in transformation. EMBO J. 6, 2053-2060.
SCHRODER, C.H., DEZAZZO, J., KNOPF, K.W., KAERNER, H.C., LEVINE, M., and GLORIOSO, J. (1985). A herpes simplex virus type 1 mutant with a deletion in the polypeptide coding sequences of the ICP4 gene. J. Gen. Virol. 66, 1589-1593.
SCHULE, R., MULLER, M., KALTSCHMIDT, C. and RENKAWITZ, R. (1988b). Many transcription factors interact synergistically with steroid receptors. Science 242, 1418-1420.
SCHULE, R., MULLER, M., OTSUKA-MURAKAMI, H. and RENKAWITZ, R. (1988a). Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. Nature 332, 87-90.
SEARS, A.E., HALLIBURTON, I.W., MEIGNIER, B., SILVER, S., and ROIZMAN, B. (1985). Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression in permissive and restricted cells and establishment of latency in mice. J. Virol. 55, 338-346.
SEKULOVICH, R.E., LEARY, K. and SANDRI-GOLDIN, R.M. (1988). The herpes simplex virus alpha protein ICP27 can act as a trans-repressor or a trans-activator in combination with ICP4 and ICP0. J. Virol. 62, 4510-4522.
SHAPIRA, M., HOMA, F.L., GLORIOSO, J.C. and LEVINE, M. (1987). Regulation of the herpes simplex virus type 1 late (gamma) glycoprotein C gene: sequences between base pairs -34 to +39 control transient expression and responsiveness to transactivation by the products of the immediate early (alpha) 4 and 0 genes. Nucl. Acids Res. 15, 3097-3111.
SHELDRICK, P. and BERTHELOT, N. (1974). Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. USA 39, 667-678.
SHIRAKI, K., and HYMAN, R.W. (1987). The immediate early proteins of Varicella-Zoster virus. Virology 156, 423-426.
SHIRAKI, K. and RAPP, F. (1986). Establishment of herpes simplex virus latency in vitro with cycloheximide. J. Gen. Virol. 67, 2497-2500.
SHOWALTER, L.D., ZWEIG, M., and HAMPAR, B. (1981). Monoclonal antibodies to herpes simplex type 1 proteins including the immediate-early protein ICP4. Infection and Immunity 34, 684-692.
SIGLER, P.B. (1988). Acid blobs and negative noodles. Nature 333, 210-212.
SILVER, S., and ROIZMAN, B.R. (1985). Gamma -Thymidine kinase chimeras are identically transcribed but regulated as gamma$_2$ genes in herpes simplex virus genomes and as beta genes in cell genomes. Mol. Cell. Biol. 5, 518-528.
SIMON, M.C., FISCH, T.M., BENECKE, B.J., NEVINS, J.R. and HEINTZ, N. (1988). Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for Ela regulation. Cell 52, 723-729.
SKINNER, G.B.R. (1976). Transformation of primary rat embryo fibroblasts by type 2 herpes simplex virus: evidence for a hit and run mechanism. British Journal of Experimental Pathology 57, 361-376.
SLAVICEK, J.M., JONES, N.C. and RICHTER, J.D. (1988). Rapid turnover of adenovirus Ela is determined through a co-translational mechanism that requires an amino terminal domain. EMBO J. 7, 3171-3180.
SMILEY, J.R., SMIBERT, C., and EVERETT, R.D. (1987). Expression of a cellular gene cloned in herpes simplex

virus: rabbit beta globin is regulated as an early viral gene in infected fibroblasts. J. Virol. 61, 2368-2377.
SMITH, C.A. and SCHAFFER, P.A. (1987a). Mutants defective in herpes simplex virus type 2 ICP4: isolation and preliminary characterization. J. Virol. 61, 1092-1097.
SMITH, C.A. and SCHAFFER, P.A. (1987b). Intertypic recombinants of herpes simplex virus types 1 and 2 infected-cell polypeptide 4. Virology 160, 176-182.
SMITH, D.H. and ZIFF, E.B. (1988). The amino-terminal region of the adenovirus serotype 5 E1a protein performs two separate functions when expressed in primary rat kidney cells. Mol. Cell. Biol. 8, 3882-3890.
SMITH, I.L. and SANDRI-GOLDIN, R.M. (1988) Evidence that transcriptional control is the major mechanism of control for the glycoprotein D gene in herpes simplex virus type 1 infected cells. J. Virol. 62, 1474-1477.
SORGER, P.K. and PELHAM, H.R.B. (1988). Yeast heat shock transcription factor is an essential DNA binding protein that exhibits temperature dependent phosphorylation. Cell 54, 855-864.
SOUTHERN, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.
SPAETE, R.R., and FRENKEL, N. (1982). The herpes simplex virus amplicon: a new eukaryotic defective-virus cloning-amplifying vector. Cell 30, 295-304.
SPEAR, P.G. and ROIZMAN, B. (1972). Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. J. Virol. 9, 143-159.
SPEAR, P.G. and ROIZMAN, B. (1980). Herpes simplex viruses, in: "Molecular biology of tumor viruses, 2nd edition, part 2, DNA tumor viruses" pp615-745, J.Tooze (ed.), Cold Spring Harbor Laboratory, N.Y.
SPIVACK, J.G. and FRASER, N.W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection of mice. J. Virol. 61, 3841-3847.
STEINER, I., SPIVACK, J.G., O'BOYLE, D.R., LAVI, C. and FRASER, N.W. (1988). Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J. Virol. 62, 3493-3496.
STEVENS, J.G. and COOK, M.L. (1971). Latent herpes simplex virus in spinal ganglia of mice. Science 173, 843-845.
STEVENS, J.G., WAGNER, E.K., DEVI-RAO, G.B., COOK, M.L. and FELDMAN, L.T. (1987). RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurones. Science 235, 1056-1059.
STOW, N.D. (1982). Localization of an origin of DNA replication within the TR_S/IR_S repeated region of the herpes simplex virus type 1 genome. EMBO J. 1, 863-867.
STOW, N.D. (1985). Mutagenesis of a herpes simplex virus origin of DNA replication and its effect on viral interference. J. Gen. Virol. 66, 31-42.
STOW, N.D., and McMONAGLE, E.C. (1983). Characterization of the TR_S/IR_S origin of DNA replication of herpes simplex virus type 1. Virology 130, 427-438.
STOW, N.D., McMONAGLE, E.C., and DAVISON, A.J. (1983). Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Res. 11, 8205-8220.
STOW, N.D., MURRAY, M.D. and STOW, E.C. (1986). *Cis*-acting Signals Involved in the Replication and Packaging of

Herpes Simplex Virus Type-1 DNA. In Botchan,M., Grodzicker,T. and Sharp,P.A. (eds.) CANCER CELLS 4/ DNA Tumor Viruses, Cold Spring Harbor Lab., NY, pp497-507.

STOW, N.D., and STOW, E.C. (1986). Isolation and characterisation of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the Immediate-Early polypeptide Vmw110. J. Gen. Virol. 67, 2571-2585.

STRAHLE, U., SCHMID, W. and SCHUTZ, G. (1988). Synergistic action of the glucocorticoid receptor with transcription factors. EMBO J. 7, 3389-3395.

STRINGER, J.R., HOLLAND, L.E., SWANSTROM, R.I., PIVO, K. and WAGNER, S.E.K. (1977). Quantitation of herpes simplex virus type 1 RNA in infected cells. J. Virol. 21, 889-901.

STROM, T. and FRENKEL, N. (1987). Effects of herpes simplex virus on mRNA stability. J. Virol. 61, 2198-2207.

STRUHL, K. (1987). Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell 49, 295-297.

SWANSTROM, R.I., and WAGNER, E.K. (1974). Regulation of synthesis of herpes simplex type 1 virus mRNA during productive infection. Virology 60, 522-533.

SYDISKIS, R.J. and ROIZMAN, B. (1966). Polysomes and protein synthesis in cells infected with a DNA virus. Science 153, 76-78.

SYDISKIS, R.J. and ROIZMAN, B. (1967). The disaggregation of host polysomes in productive and abortive infection with herpes simplex virus. Virology 32, 678-686.

TACKNEY, C., CACHIANES, G., and SILVERSTEIN, S. (1984). Transduction of the chinese hamster ovary aprt gene by herpes simplex virus. J. Virol. 52, 606-614.

TAKAHASHI, K., VIGNERON, M. MATTHES, M., WILDEMAN, A., ZENKE, M. and CHAMBON, P. (1986). Requirements of stereospecific alignments for initiation from the simian virus 40 early promoter. Nature 319, 121-126.

TEDDER, D.G. and PIZER, L.I. (1988). Role for DNA-protein interacrion in activation of the herpes simplex virus glycoprotein D gene. J. Virol. 62, 4661-4672.

TIMBURY, M.C. and SUBAK-SHARPE, J.H. (1973).Genetic interactions between temperature-sensitive mutants of types 1 and 2 herpes simplex virus. J. Gen. Virol. 18, 347-357.

TREMBLAY, M.L., YEE, S-P., PERSSON, R.H., BACCHETTI, S., SMILEY, J.R., and BRANTON, P.E. (1985). Activation and inhibition of expression of the 72,000-Da early protein of adenovirus type 5 in mouse cells constitutively expressing an immediate-early protein of herpes simplex virus type 1. Virology 144, 35-45.

TRIEZENBERG, S.J., LaMARCO, K.L and McKNIGHT, S.L. (1988b). Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes and Dev. 2, 730-742.

TRIEZENBERG, S.J., KINGSBURY, R.C. and McKNIGHT, S.L. (1988a). Functional dissection of VP16 the trans-activator of herpes simplex virus immediate early gene expression. Genes and Dev. 2, 718-729.

UMENE, K. (1986). Conversion of a fraction of the inverted repeats in the S component of the herpes simplex virus type 1 genome. J. Gen. Virol. 67, 1035-1048.

VAHLNE, A., SVENNERHOLM, B. and LYCKE, E. (1979). Evidence for herpes simplex type-selective receptors on cellular plasma membranes. J. Gen. Virol. 44, 217-225.

VAN DYKE, M.W., ROEDER, R.G. and SAWADOGO, M. (1988). Physical analysis of transcription preinitiation complex assembly on a class II gene promoter. Science 241, 1335-1338.

VELCICH, A. and ZIFF, E. (1985). Adenovirus Ela proteins repress transcription from the SV40 early promoter. Cell 40, 705-716.

VELCICH, A. and ZIFF, E. (1988). Adenovirus Ela ras cooperation activity is separate from its positive and negative transcription regulatory functions. Mol. Cell. Biol. 8, 2177-2183.

VLAZNY, D.A. and FRENKEL, N. (1981). Replication of herpes simplex virus DNA: localization of replication signals within defective virus genomes. Proc. Natl. Acad. Sci. USA 78, 742-746.

VLAZNY, D.A., KWONG, A. and FRENKEL, N. (1982). Site specific cleavage and packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full length viral DNA. Proc. Natl. Acad. Sci. USA 79, 1423-1427.

WADSWORTH, S., JACOB, R.J. and ROIZMAN, B. (1975). Anatomy of herpes simplex virus DNA. II. Size composition and arrangement of inverted terminal repeats. J. Virol. 15, 1487-1497.

WAGNER, E.K. (1985). Individual HSV Transcripts: Characterisation of Specific Genes. In: "The Herpesviruses" B.Roizman (ed.), Plenum Press, New York/London. 3, pp45-104.

WAGNER, E.K., FLANAGAN, W.M., DEVI-RAO, G., ZHANG, Y.-F., HILL, J.M., ANDERSON, K.P. and STEVENS, J.G. (1988). The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. 62, 4577-4585.

WATSON, R.J. and CLEMENTS, J.B. (1978). Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. Virology 91, 364-379.

WATSON, R.J., and CLEMENTS, J.B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature 285, 329-330.

WEBER, P.C., LEVINE, M. and GLORIOSO, J.C. (1987). Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science 236, 576-579.

WEBSTER, N., JIN, J.R., GREEN, S., HOLLIS, M. and CHAMBON, P. (1988). The yeast UAS_G is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans-activator. Cell 52, 169-178.

WECHSLER, S.L., NESBURN, A.B., WATSON, R., SLANINA, S.M. and GHIASI, H. (1988). Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distint latency related RNAs containing open reading frames. J. Virol. 62, 4051-4058.

WEINBERGER, C., THOMPSON, C.C., ONG, E.S., LEBO, R., GRUOL, D.J. and EVANS, R.M. (1986). The c-erbA gene encodes a thyroid hormone receptor. Nature 324, 641-646.

WEINHEIMER, S.P., and McKNIGHT, S.L. (1987). Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. 195, 819-833.

WELLER, S.K., CARMICHAEL, E.P., ASCHMAN, D.P., GOLDSTEIN, D.J. and SCHAFFER, P.A. (1987). Genetic and phenotypic characterization of mutants in four essential genes that map to the left half of the HSV-1 U_L DNA. Virology 161,

198-210.
WELLER, S.K., SPADARO, A., SCHAFFER, J.E., MURRAY, A.W., MAXAM, A.M. and SCHAFFER, P.A. (1985). Cloning, sequencing, and functional analysis of ori_L, a herpes simplex virus type 1 origin of DNA synthesis. Mol. Cell. Biol. 5, 930-942.
WHITLEY, R.J. (1985). Epidemiology of herpes simplex viruses, in: "The herpesviruses" vol. 3, ppl-44, B.Roizman (ed.), Plenum Press, N.Y.
WHYTE, P., BUCH-KOVICH, K.J., HOROWITZ, J.M., FRIEND, S.H., RAYBUCK, M., WEINBERG, R.A. and HARLOW, E. (1988b). Association between an oncogene and an anti-oncogene: the adenovirus Ela proteins bind to the retinoblastoma gene product. Nature 334, 124-128.
WHYTE, P., RULEY, H.E. and HARLOW, E. (1988a). Two regions of the adenovirus early region la protein are required for transformation. J. Virol. 62, 257-265.
WIGDAHL, B.L., SCHECK, A.C., DeCLERQ, E. and RAPP, F. (1982). High efficiency latency and activation of herpes simplex virus in human cells. Science 217, 1145-1146.
WILCOX, K.W., KOHN, A., SKLYANSKAY, E., and ROIZMAN, B. (1980). Herpes simplex virus phosphoproteins I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. 33, 167-182.
WILDEMAN, A.G., ZENKE, M., SCHATZ, C., WINTZERITH, M., GRUNDSTOM, T., MATTHES, H., TAKAHASHI, K. and CHAMBON, P. (1986). Specific protein binding to the simian virus 40 enhancer in vitro. Mol. Cell. Biol. 6, 2098-2105.
WILDY, P., SMITH, C., NEWTON, A.A. and DENDY, P. (1961). Quantitative cytological studies of HeLa cells infected with herpesvirus. Virology 15, 486-500.
WILDY, P., RUSSELL, W.C. and HORNE, R.W. (1960). The morphology of herpes viruses. Virology 12, 204-222.
WILKIE, N.M. (1973). The synthesis and substructure of herpesvirus DNA: the distribution of alkali labile single strand interuptions in HSV-1 DNA. J. Gen. Virol. 8, 525-533.
WILKIE, N.M. (1976). Physical maps for herpes simplex virus type 1 DNA for restriction endonucleases HindIII, HpaI and X.bad. J. Virol. 20, 222-233.
WILLIAMS, M.V. (1988). Herpes simplex virus-induced dUTPase: target site for antiviral chemotherapy. Virology 166, 262-264.
WILLMANN, T. and BEATO, M. (1986). Steroid-free glucocorticoid receptor binds specifically to mouse mammary tumour virus DNA. Nature 324, 688-691.
WINGENDER, E. (1988). Compilation of transcription regulating proteins. Nucleic Acids Res. 16, 1879-1902.
WIRTH, T. and BALTIMORE, D. (1988).Nuclear factor $NF-_kB$ can interact functionally with its cognate site to provide lymphoid specific promoter function. EMBO J. 7, 3109-3113.
WORKMAN, J.L., ABMAYR, S.M., CROMLISH, W.A. and ROEDER, R.G. (1988). Transcriptional regulation by the immediate early protein of pseudorabies virus during in vitro nucleosome assembly. Cell 55, 211-219.
WORRAD, D.M. and CARADONNA, S. (1988). Identification of the herpes simplex virus uracil-DNA glycosylase. J. Virol. 62, 4774-4777.
WU, C.A., NELSON, N.J., McGEOCH, D.J. and CHALLBERG, M.D. (1988).Identification of herpes simplex virus type 1 genes required for origin dependent DNA synthesis. J. Virol. 62, 435-443.

WU, L., ROSSER, D.S.E., SCHMIDT, M.C. and BERK, A. (1987). A TATA box implicated in E1a transcriptional activation of a simple adenovirus 2 promoter. Nature 326, 512-515.

WU, L. and BERK, A.J. (1988). Transcriptional activation by the pseudorabies virus immediate early protein requires the TATA box element in the adenovirus E1b promoter. Virology 167, 318-322.

YAGER, D.R. and COEN, D.M. (1988). Analysis of the transcript of the herpes simplex virus DNA polymerase gene provides evidence that polymerase expression is inefficient at the level of translation. J. Virol. 62, 2007-2015.

YEE, A.S., REICHEL, R., KOVESDI, I. and NEVINS, J.R. (1987). Promoter interaction of the E1a inducible factor E2F and its potential role in the formation of a multicomponent complex. EMBO J. 6, 2061-2068.

YEE, S.-P. and BRANTON, P.E. (1985). Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. Virology 174, 142-153.

ZEHRING, W.A., LEE, J.M., WEEKS, J.R., JOKERST, R.S. and GREENLEAF, A.L. (1988). The C-terminal repear domain of RNA polymerase II largest subunit is essential *in vivo* but is not required for accurate transcription initiation *in vitro*. Proc. Natl. Acad. Sci. USA 85, 3698-3702.

ZENKE, M., GRUNSTROM, T., MATTHES, H., WITZERITH, M., SCHATZ, C., WILDEMAN, A. and CHAMBON, P. (1986). Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. 5, 387-397.

ZENKE, M., KAHN, P., DISELA, C., VENNSTROM, B., LEUTZ, A., KEEGAN, K., HAYMAN, M.J., CHOI, H.R., YEW, N., ENGEL, Y.D. and BEUG, H. (1988). v-*erbA* specifically suppresses transcription of the avian erythrocyte anion transporter (band 3) gene. Cell 52, 107-119.

ZERLER, B., ROBERTS, R.J., MATHEWS, M.B. and MORAN, E. (1987). Different functional domains of the adenovirus E1a gene are involved in regulation of host cell cycle products. Mol. Cell. Biol. 7, 821-829.

ZHANG, Y.-F. and WAGNER, E.K. (1987). The kinetics of expression of individual herpes simplex virus type 1 transcripts. Virus Genes 1, 49-60.

ZHENG, X.-M., MONCOLLIN, V., EGLY, J.-M. and CHAMBOM, P. (1987). A general transcription factor forms a stable complex with RNA polymerase B (II). Cell 50, 361-368.

ZHU, L. and WELLER, S.K. (1988). UL5, a protein required for HSV DNA synthesis: genetic analysis, overexpression in *Escherichia coli*, and generation of polyclonal antibodies. Virology 166, 366-378.

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The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation

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ABSTRACT

The immediate-early (IE) protein Vmw175 (ICP4) of HSV-1 is required for the transcription of later classes of viral genes and the repression of IE gene expression. We have previously constructed a panel of plasmid-borne insertion and deletion mutants of the gene encoding Vmw175 and assayed their ability to regulate transcription in transient transfection assays. By this approach we have mapped the regions of the Vmw175 amino acid sequence that are required for transcriptional activation and repression of herpes virus promoters. This paper describes the use of nuclear extracts, made from cells transfected with these mutant plasmids, in gel retardation DNA binding assays in order to define the regions of Vmw175 involved in binding to a specific Vmw175 DNA binding site. The results show that amino acid residues 275-495 (a region which is highly conserved between Vmw175 and the varicella-zoster virus "IE" 140K protein) include structures which are critically required for specific DNA binding, transactivation and repression. This raises the interesting paradox that although the specific DNA sequence recognized by Vmw175 is not commonly found in its target promoters, the protein domain required for recognition of this sequence is required for promoter activation.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a large complex virus containing a double stranded DNA genome of around 150kb. Upon infection of tissue culture cells the genes of HSV-1 are expressed in a sequential cascade of three broad temporal classes, defined as immediate-early (IE), early and late (1). Expression of IE proteins is required for transcription of later classes of genes (2,3,4) and there is evidence that at least four of the five IE gene products are regulators of viral gene expression (reviewed, 5). However, characterization of temperature-sensitive (ts) and deletion mutations in these five genes has revealed that only Vmw175 (ICP4) and Vmw63 (ICP27)

(the products of IE genes 3 and 2) are essential for virus growth in cell culture (6-12). Functional Vmw175 is required for the transcriptional activation of early and late genes and also the repression of IE genes (13). Both of these activities can be studied using cloned copies of IE gene 3 in transient transfection assays to transactivate viral early gene promoters or to repress expression from its own promoter (5). Transactivation and repression have also been reproduced *in vitro* using partially purified Vmw175 (14,15).

Vmw175 is a large, phosphorylated, nuclear protein (16) which can be isolated as a homodimer (17). Whilst most of the IE proteins bind to DNA to some extent *in vitro* (18) Vmw175 is the only one known to interact with specific target sequences which include a proposed consensus binding sequence ATCGTC (15,19). Vmw175 has been shown to bind to this sequence in the promoter regions of the IE1, IE3 and glycoprotein gD genes (19-22) but has also been reported to bind to several viral DNA fragments which lack the consensus (20,23). Evidence from a variety of laboratories suggests a correlation between the ability of Vmw175 to bind ATCGTC at the transcriptional start site of IE gene 3, and autoregulation of this promoter. However, less is known about the mechanism by which Vmw175 transactivates promoters. Most such promoters lack sequences closely related to the consensus binding site, and indeed there is no evidence for any transactivation specific promoter sequences being necessary for the action of Vmw175 (24-26). Recent evidence suggests that Vmw175 can also bind to alternative sites not related to the consensus binding-site, both in promoter regions and the 5' transcribed, non-coding regions of late genes (23); the significance of this is not yet known.

IE gene 3 is present in two copies in HSV-1, being carried on the repeat sequences bounding the short unique region of the genome (27). The sequence of the gene reveals an uninterrupted open reading frame encoding a protein of 1298 amino acids having a predicted unmodified molecular weight of 133kDa (28). The protein is homologous to a predicted 140kDa protein of a related alphaherpesvirus, varicella-zoster virus (VZV) (28,29). The VZV 140K product is also a potent activator of transcription (24,30,

unpublished results) and can complement HSV-1 mutants with ts lesions in Vmw175 (31,32). On the basis of the sequence homology between the two proteins, and various other criteria, the Vmw175 sequence has been divided into five structural regions, with regions 2 and 4 containing the main regions of homology with the VZV protein (28,29) (see Figure 3).

We have previously described the construction and functional analysis of a large number of small, in-frame, insertion and deletion mutants of a plasmid-borne copy of the gene encoding Vmw175 (33). By studying the activity of the resultant mutant polypeptides in transient transfection assays we have defined the regions of the protein which are important for repression of its own promoter, and those important for transactivation of the promoter of the HSV early gene encoding glycoprotein gD, in the presence of another HSV-1 IE transactivator, Vmw110. Independent analyses performed by DeLuca and Schaffer (34,35) using chain-termination mutants of the gene encoding Vmw175 in both plasmids and recombinant HSV-1 virus have defined similar functional domains.

This paper presents the results from further analyses of plasmid-borne insertion and deletion mutants in Vmw175. Extracts were made from transfected cells and used in gel retardation assays to measure the ability of each mutant polypeptide to bind to a DNA probe containing the consensus Vmw175-binding site at the transcription start site of IE gene 3. The results show that the regions of Vmw175 required for DNA binding closely correspond to those involved in transcriptional regulation. In particular, a region crucial for site specific DNA binding lies within amino acids 275-495.

MATERIALS AND METHODS

Plasmids. Plasmid p175 expresses Vmw175 under the control of the SV40 early promoter and enhancer (36). Plasmids p111 (36) and p63 (37) express other HSV-1 IE gene products, Vmw110 and Vmw63. pGX38 carries the gene encoding the major DNA binding protein, ICP8. The insertion mutant series pI1-39 was derived from p175 by the insertion of 12bp EcoRI linkers at random restriction sites and the deletion mutants were derived from these (33). Deletions pD13, pD14 and pD15 were created by recombining pairs of insertion mutants at the inserted EcoRI linker to generate inframe deletions (see Table 2).

Cell culture, infection and transfection. HeLa cells were obtained from the ATCC through Flow Laboratories and grown in Glasgow Modified Eagle's Medium containing 10% foetal calf serum. The viruses used were derived from HSV-1 strain 17 syn$^+$ (38). Cells were infected at a multiplicity of infection of 5pfu per cell and nuclear extracts made 5h after a 1h adsorption period. Cells on 90 or 135mm plates (3 or 7x10^6 cells per plate) were transfected by the procedure of Corsalo and Pearson (39). In some recent transfections HBS was replaced by the more efficient BBS (40). 30 or 60ug of p175 derived plasmids were used plus 15 or 30ug of pUC9 as carrier. Nuclear extracts were made 45-50h post transfection.

Preparation of nuclear extracts. Nuclear extracts of infected and transfected cells were prepared by a procedure modified from that of Dignam et al.,(41). Cells scraped from 135 or 90mm culture dishes were washed in phosphate buffered saline (PBS) and resuspended in 2 volumes buffer A plus 0.5% NP40 (10mM Hepes pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT). After 10 minutes lysis on ice nuclei were pelleted by successive 2krpm/10 minute and 12krpm/20 minute spins in the Sorvall SS34 rotor. Proteins were eluted from the nuclei by incubating for 30 minutes on ice in 2 volumes buffer C (20mM Hepes pH7.9, 25% glycerol, 0.42mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT) before clearing with a 30 minute 15krpm spin. The supernatants were flash frozen and stored at -170^{o}C.

Some whole cell extracts were produced by lysing cells with buffer C plus 0.5% NP40; the results using extracts made by the two methods were generally indistinguishable.

Determination of the relative quantities of Vmw175 in nuclear extracts. Nuclear extracts were assayed by the ELISA technique in order to determine the relative amount of Vmw175 present. Microtitre wells were coated overnight with extract (5ul) plus 100ul of a solution containing 0.05% sodium azide, 1% ovalbumin, 1% BSA, 0.1% Tween 20 in PBS. 100ul of a 1/5000 dilution of the monoclonal antibody 58S (kindly supplied by M.Zweig, 42) was adsorbed to the coated wells by shaking for 1h at 37^{o}C then 100ul of a 1/3000 dilution of BIO-RAD horseradish peroxidase labelled goat anti-mouse IgG was bound to this at 20^{o}C for 1h. Extensive washes with PBS/0.1% Tween 20 were carried out between all steps. The absorbance at 405nm was measured 10 minutes after addition of substrate.

Gel retardation assay. The binding of Vmw175 to the IE gene 3 cap site was assayed as described by Muller (22) using the gel retardation DNA binding assay (43). The 45bp Ava I/BamHI DNA fragment spanning the IE3 cap site (-17 to +27) was end labelled by filling in using T4 DNA polymerase, separated on an 8% polyacrylamide gel, eluted and purified through a Sephadex G-50 column. Binding reactions were generally carried out at 20^{o}C for 20 minutes in a 20-30ul mix containing 1ug poly(dI).poly(dC), about 0.1ng probe (2000cpm) and 1-4ul nuclear extract (4-20ug protein) in 10mM Tris.HCl pH7.6, 1mM EDTA, 0.1% NP40. The variation in final salt concentration (20-80mM NaCl) did not affect the formation of the specific Vmw175 complex, which is stable up to at least 300mM NaCl (results not shown). The complexes were resolved on 4% polyacrylamide gels, run in 0.5xTBE buffer, which were dried before autoradiography.

Antibody shifts were carried out by incubating the binding reaction for an additional 10-15 minutes after adding lul of a 1/100 dilution of monoclonal antibody 58S.

RESULTS

Vmw175 in nuclear extracts made from transfected cells forms an authentic complex with the IE gene 3 cap site.

We have used a gel retardation assay similar to that of Muller (22) to show that nuclear extracts made from HeLa cells transfected with a plasmid expressing HSV-1 Vmw175 form a Vmw175 specific complex with the IE3 cap site which is essentially identical to that formed using extracts of virus infected cells. Using a short end-labelled DNA probe spanning the cap site of this gene a single major, viral specific, retarded complex is detected on non-denaturing polyacrylamide gels after incubation of the probe with infected cell nuclear extract for 20 minutes at 20°C (Figure 1A, tracks 3 and 4). This complex is further retarded after additional incubation with the Vmw175 specific monoclonal antibody 58S (42) which recognizes an epitope near to the C-terminus of Vmw175 (33). These complexes were not formed using extracts made from mock infected cells nor from cells infected with an HSV-1 mutant which produces no Vmw175, in1411 (44) (Figure 1A). The complex was also formed by extracts made from cells infected with dl1403, a mutant lacking both copies of IE gene 1 which encodes Vmw110 (8).

An apparently viral specific complex, labelled 'z', of much greater mobility was occasionally formed (Figure 1B), especially if the binding reaction was carried out at elevated temperatures. This complex was not further retarded by the antibody 58S, indicating that it might be formed by a protein unrelated to Vmw175, or, by a proteolytic fragment of Vmw175 lacking the C-terminal 58S epitope.

Extracts made from cells transfected with plasmid p175, which expresses HSV-1 Vmw175, also formed a complex which was usually indistinguishable from the viral specified complex and which also contained Vmw175 (Figure 2A). However, transfected cell extracts occasionally formed two specific complexes which were both recognized by 58S (Figure 2B). The smaller complex could be formed by an abnormally processed form of Vmw175 or it

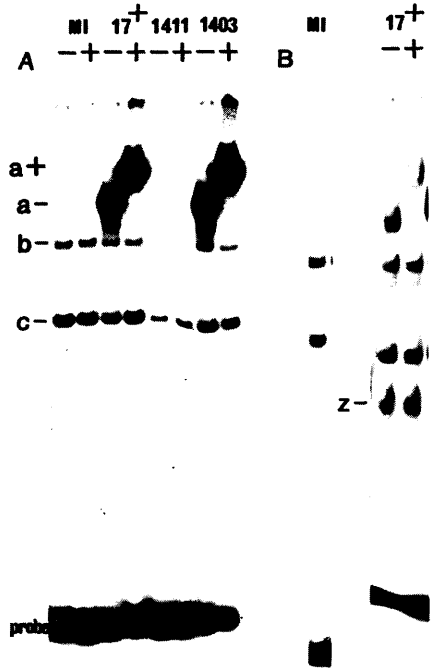

Figure 1: Autoradiograph of a gel retardation experiment showing the protein-DNA complexes formed using nuclear extracts of HSV-1 infected cells bound to a DNA probe spanning bases -17 to +27 of the HSV-1 IE gene 3. Assays were performed as described in the materials and methods, with (+) or without (-) addition of monoclonal antibody 58S. Assays perfomed at (A) 20°C or (B) 40°C. MI= mock infected, 17^+= HSV-1 strain 17^+, 1403= HSV-1 mutant dl1403, 1411= HSV-1 mutant in1411, a= retarded complex containing Vmw175, a^+= complex a further retarded with antibody, b and c are complexes containing host factors, z= novel retarded band, probe= unbound DNA probe.

might lack an unknown factor. It usually appeared when the overall efficiency of the transfections was poor and could reflect a lower level of Vmw175 in the transfected cells.

That cells transfected with p175 formed a complex identical to that formed with infected cell extracts indicates that Vmw175 is the only viral protein present in the complex. No other HSV proteins could be detected in the complex using monoclonal antibodies (against the major DNA binding protein) or antipeptide antisera (against Vmw110 and Vmw63, results not shown). The

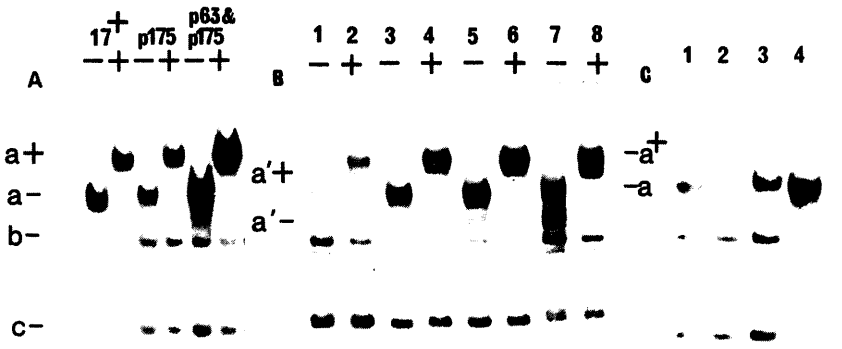

Figure 2: Nuclear extracts containing Vmw175 expressed in cells transfected with plasmid p175 form an apparently identical DNA complex to that formed by Vmw175 present in infected cell extracts. (A) Binding assays were carried out as described in Figure 1 using extracts of cells infected or transfected as shown. Unbound probe has been cut off the bottom of the portion of the gel shown. Plasmids used express Vmw175 (p175), Vmw110 (p111), Vmw63 (p63) and ICP8 (pGX38). (B) cells were transfected with p175 and the following plasmids: lanes 1 & 2, p111; 3 & 4, p111 and p63; 5 & 6, p111, p63 and pGX38; 7 & 8, p175 alone. a'= secondary complex, shifted to a'$^{+}$ with antibody. (C) Addition of extracts containing Vmw63 does not enhance Vmw175 DNA binding. Lane 1, p175; lane 2, p63; lane 3 mix of extracts used in 1 & 2, lane 4, cotransfection of p175 and p63.

presence of other HSV-1 genes in the transfection had no effect on the nature of complex formed, but addition of the gene encoding Vmw63 did increase its amount (Figures 2A,B). However, Vmw63 itself did not form a complex with this DNA probe, and the addition of an extract containing Vmw63 to one containing Vmw175 did not enhance binding (Figure 2C). Rather the effect of Vmw63 seemed to be on expression of Vmw175, as the extract made from Vmw63 and 175 encoding genes transfected together had 2.5 fold more Vmw175 than the extract from cells transfected with p175 alone, as determined by ELISA.

The regions of Vmw175 important for DNA binding closely correspond to those important for transcriptional regulation.

1. Analysis of insertion mutants. We have previously constructed a series of derivatives of p175 with twelve base pair insertions

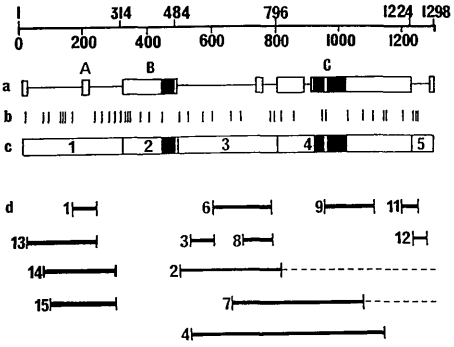

Figure 3: Map of the Vmw175 coding sequence showing the position of the insertion and deletion mutants used in this study. (a) Homology map of Vmw175 in relation to VZV 140K. The three main homology regions A, B and C are indicated. Clearly homologous regions are shown as boxes, with the most homologous sections filled (at least 80% identity). (b) Position of each linker insertion (I1-39) is represented by a vertical line. (c) Colinear map showing the 5 regions of Vmw175. (d) Extent of deletions used in this study, dotted lines represent out of frame sequences. The scale is given in amino acid residues.

at random sites in the Vmw175 coding sequence (Figure 3) and used these to define the regions of the protein that are important for transactivation of an HSV-1 early promoter and repression of the viral IE3 promoter (33). We have now investigated the DNA binding activity of these mutants by using nuclear extracts made from HeLa cells transfected with each of these 39 plasmids in gel retardation assays.

Nuclear extracts were prepared from $7x10^6$ transfected cells and one twentieth (4ul) of each was incubated with the IE3 cap site probe in a 30ul reaction volume at 20^oC for 20 minutes, then further incubated with 1ul 1/100 dilution of monoclonal 58S prior to separation on 4% polyacrylamide gels. The resulting autoradiographs are shown in Figure 4. The antibody shift was carried out to clearly resolve the Vmw175 complex from the nonspecific complexes (b) and (c) although in some cases the addition of antibody seemed to stabilize the degree of complex formation (results not shown). In addition to the major (a^+) complex, the minor (z) complex (Figure 1B) was also occasionally

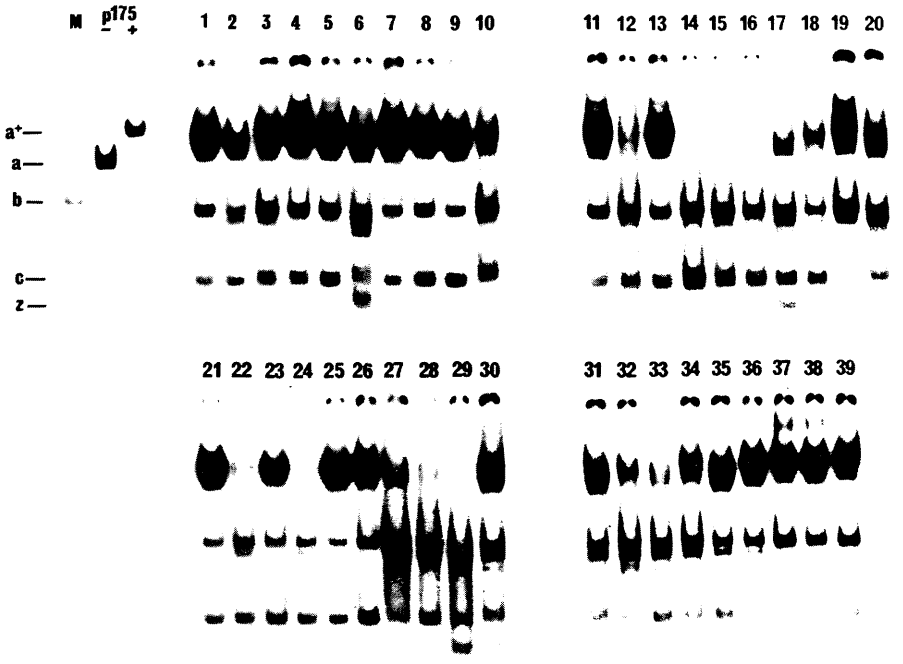

Figure 4: Binding assays using extracts of cells transfected independantly with each of the 39 insertion mutants pI1-pI39. All assays were incubated with antibody 58S except M (mock transfected) and p175/- (positive control). Complexes a, a^+, b, c and z are as described in Figure 1.

present. The binding reactions were carried out at 20°C; at higher temperatures the binding of wild type Vmw175 was significantly reduced (results not shown) although temperature sensitive mutants can be studied in this manner (unpublished results).

The amount of Vmw175 in each extract was measured by ELISA and compared to the p175 control transfection to ensure that the transfections were working with comparable efficiency and that the Vmw175 polypeptide was present in the extract. These results, together with a summary of the binding results in Figure 4, are presented in Table 1, which also summarizes the map

Table 1. A Summary of the Positions and Activities of the Insertion Mutants.

Mutant	Insert Site[a]	Transact. activity[b] x (SE)	Repress. activity[c]	DNA binding activity[d]	Relative amount of Vmw175[e]
pI1	12	35.1 (11.7)	+++	+++	288
pI2	71	53.4 (13)	++	++	203
pI3	83	49.6 (6.7)	++	+++	88
pI4	123	65.6 (12.8)	++	+++	241
pI5	130	43.8 (6.5)	+++	+++	106
pI6	137	54.4 (11.8)	++	+++	103
pI7	161	44.5 (10.5)	+++	+++	297
pI8	229	45.7 (8)	+++	+++	127
pI9	252	127 (43.2)	+++	+++	263
pI10	275	25.8 (4.6)	-	++	142
pI11	292	30.8 (6.3)	++	+++	276
pI12	310	20.0 (4.4)	+	+/-	97
pI13	324	10.0 (2.9)	-	+++	226
pI14	329	4.9 (0.9)	-	-	38
pI15	337	4.5 (0.9)	+	-	18
pI16	373	16.5 (2.8)	-	-	18
pI17	398	11.8 (1.9)	-	+	39
pI18	438	15.5 (4.4)	-	+	65
pI19	494	49.2 (10.4)	+++	+++	170
pI20	518	47.2 (10.2)	++	+++	85
pI21	561	43.4 (4.2)	+++	+++	294
pI22	591	26.2 (6.2)	++	+/-	206
pI23	649	23.9 (5.6)	++	++	297
pI24	681	21.1 (4.2)	++	+/-	91
pI25	774	38.2 (6.1)	+++	+++	291
pI26	787	80.9 (18.5)	+++	+++	70
pI27	803	45.9 (9.6)	++	+	126
pI28	843	10.8 (3.9)	+++	+/-[f]	15[f]
pI29	934	3.4 (0.5)	+	-[f]	0[f]
pI30	943	48.2 (12.3)	++	+++	58
pI31	1021	22.4 (3.7)	+++	+++	100
pI32	1066	10.3 (3.0)	+++	+	91
pI33	1098	30.0 (8.9)	++	+	15
pI34	1133	20.8 (2.3)	++	++	27
pI35	1139	48.2 (13.6)	++	+++	121
pI36	1195	86.9 (18)	+++	+++	218
pI37	1231	47.5 (10.3)	+++	+++	221
pI38	1236	63.1 (11.2)	+++	+++	245
pI39	1239	51.1 (8.6)	+++	+++	248

a The site (amino acid) of linker insertion.

b The activation of the gD promoter (using pgDCAT in conjunction with Vmw110 provided by p111) is given as a percentage of that obtained in parallel experiments with p175. The mean of at least four independent determinations is given, with the standard error of the mean. Wild type p175 activated pgDCAT expression 20-fold over uninduced levels (33).

c The qualitative ability to repress the IE3 promoter of pIE3CAT compared to p175 in cotransfection titration experiments. Repression activity is scored from - (essentially no activity) to +++ (wild type activity) (33).

d The ability of nuclear extracts made from HeLa cells transfected with each of the mutants to bind to the IE3 cap site. Binding activity is scored from visual inspection of Figure 4: - no binding, +/- barely detectable, + to +++ increasing binding activity.

e Relative amount of Vmw175 in each nuclear extract determined by ELISA. (p175=100)

f Further experiments with this mutant did produce detectable amounts of pI29 Vmw175 as described in the text and Figure 5.

positions and earlier transcriptional regulation results published for these plasmids (33).

Table 1 shows that mutants impaired in their ability to bind to the IE3 cap site in vitro broadly correspond to those impaired in their ability to transactivate and to repress transcription in transfection assays. For example insertion mutants pIl2 to pIl8 (with the exception of pIl3) are all significantly reduced in the transactivation and repression assays and also in their ability to bind to the IE3 cap site. This is especially true of mutants pIl4-pIl8. Indeed it is clear that this portion of the protein, (region 2; amino acids 315-484) is of critical importance for both transcriptional control and DNA binding. Only one mutant in region 2, pIl3, can efficiently bind to this DNA probe, but fails to express either repression or transactivation activity.

Insertion mutants throughout the majority of region 1 are virtually unimpaired in DNA binding activity or transcriptional regulation. However, two mutants, pIl0 and pIl2, situated at the end of region 1 close to the junction with region 2, are significantly reduced in their abilty to repress the IE3 promoter, and both are impaired in their ability to bind to the IE3 cap site, pIl2 markedly so.

However, DNA binding does not show an absolute correlation with either transactivation or repression. For example two insertion mutants in region 3, pI22 and pI24, are moderately efficient transcriptional regulators, but bind to the IE3 promoter very poorly in vitro. Similarly several mutants in region 4 have markedly reduced DNA binding activity, and whilst some of these have deficient transactivation activity (pI28, pI29 and pI32) all except pI29 are fairly efficient repressors of IE3 expression. These individual descrepancies in the correlation between DNA binding and transcriptional activity may reflect differences between binding in vitro and in the nucleus of the transfected cell.

One mutant, pI29, which failed to bind DNA in Figure 4 failed to produce detectable Vmwl75 in these initial nuclear extracts. However, previous studies had detected low numbers of cells expressing nuclear Vmwl75 by immunofluorescence of cells transfected with pI29. When the transfections were repeated

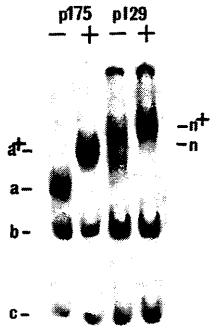

Figure 5: An abnormal complex is formed by total cell extracts of cells transfected with pI29. Binding is performed at 0°C with (+) or without (-) addition of antibody 58S using whole cell extracts of cells transfected with pl75 or pI29. Complexes a, a^+, b and c are as described in Figure 1, the novel complex, n, formed by pI29 transfected extracts is shifted to n^+ by addition of antibody.

using BBS instead of HBS, detectable amounts of Vmwl75 could be recovered and shown to bind to the IE3 cap site probe when incubated at 0°C (Figure 5). However, the complex formed was of reduced mobility both in the presence and absence of antibody. This may indicate that the I29 mutation induces incorrect processing or folding of Vmwl75. Total cell extracts of cells transfected with pI29 had greater amounts of Vmwl75 binding activity than nuclear extracts which may indicate that the polypeptide encoded by pI29 is deficient in nuclear localization.

It is also possible that the protein expressed by pI29 is unstable either *in vivo* or *in vitro*. The clustering of insertion sites which result in plasmids producing reduced amounts of Vmwl75 in nuclear extracts (Table 1) may indicate that certain regions are important for protein stablity, for example the regions of the protein defined by insertions I14-18, I28-30 and I33-34. Interestingly, mutations in the main DNA binding domain (I14-18) may result in a less stable protein. Apparently low levels of protein expression do not of themselves explain poor DNA binding since some extracts containing low levels of ELISA-detectable protein are capable of normal DNA binding, for

Table 2. A Summary of the Extent and Activities of the Deletion Mutants.

Mutant	Deleted amino acids	Frame[a]	IIF[b]	Transact. act.[c] x (SE)	Repress. act.[d]	DNA binding act.[e]	Relative amount of Vmw175[f]
pD13	12-229	IN	n.d.[g]	n.d.	n.d.	weak	25
pD14	72-292	IN	n.d.	n.d.	n.d.	-	14
pD15	84-292	IN	n.d.	n.d.	n.d.	-	15
pD1	162-229	IN	nuc.	30 (4.8)	+++	+	216
pD2	495-803	OUT	-	23 (4.0)	-	-	n.d.
pD2F	496-803	IN	cyt.	5 (0.5)	+	weak	52
pD3	518-591	IN	nuc.	4 (0.5)	+++	-	107
pD4	518-1133	IN	cyt.	18 (3.3)	++	-	27
pD5	562	OUT	-	3 (1.3)	++	-	n.d.
pD6	592-774	IN	cyt.	29 (2.8)	+	weak	25
pD7	650-1066	OUT	-	27 (5.4)	-	-	n.d.
pD7F	650-1066	IN	cyt.	10 (1.5)	+	-	88
pD8	682-774	IN	cyt.	57 (5.6)	-	weak	95
pD9	934-1098	IN	nuc.	4 (0.6)	+++	+	13
pD11	1196-1239	IN	nuc.	6 (2.2)	+++	+	59
pD12	1232-1269	IN	nuc.	7 (2.2)	++	+	52

a Phase of reading frame 3' to deletion
b Cellular localization of protein as determined by immunofluorescence (33). nuc. nuclear, cyt. cytoplasmic, - not detectable by immunofluorecence
c The activation of the gD promoter as described in Table 1 (and ref. 33).
d The qualitative ability to repress the IE3 promoter as described for Table 1 (and ref. 33).
e The ability of whole cell extracts made from cells transfected with each of the mutants to bind to the IE3 cap site probe in gel retardation assays (at 0°C). Binding is scored simply as positive (+), negative (-) or weak.
f Relative amount of Vmw175 in each nuclear extract determined by ELISA. (p175=100, the results are given for the extracts used in Figure 6, or, in the case of the non-binding mutants, for extracts containing the most Vmw175; out of frame proteins could not be detected by ELISA.)
g n.d. not done

example pI34 and the deletion mutant pD9 (Table 2). It is also possible that the low ELISA measurements could be explained by failure to efficiently extract or detect mutant polypeptides if, for example, insertions effect protein solubility or expression of the 58S epitope.

2. Analysis of deletion mutants. We have previously described the construction of a number of in frame deletion mutants within the Vmw175 coding region using the inserted EcoRI sites (33).

These mutants were assayed for their ability to bind to the IE3 DNA probe at 0°C in order to increase the sensitivity of the assay. These results are presented in Table 2 with the previously published results from transcription and immunofluorescence assays and the relative amount of ELISA detectable protein in each extract. Transfections were repeated at least three times for those extracts which failed to bind DNA and the highest ELISA measurement recorded.

DeLuca and Schaffer (35) have previously reported that a truncated polypeptide, encoding only the first 590 amino acids of Vmw175, is able to bind to DNA under some conditions. The results presented in Table 2 and Figure 6 confirm their results and allow us to map more finely the regions of Vmw175 unimportant for DNA binding.

Plasmids which expressed variants of Vmw175 with some DNA binding activity all have deletions which do not include amino acids 229-495, (Table 2, Figure 6). Whilst not all plasmids with deletions lying totally outwith this region express binding competent proteins (for example pD3, pD5 and pD7F) individual deletions which do not completely abolish DNA binding cover most of the rest of the gene (deletions 1, 2F, 6, 8, 9, 11, 12 and 13; Table 2). Because large deletions of the protein may cause many unpredictable changes in protein stability and conformation, perhaps only those deletions which do not totally abolish DNA binding activity should be considered significant results. The results with the deletion mutants, therefore, confirm the conclusion from the insertion mutant assays that the main DNA binding domain of Vmw175 centres around region 2.

The correlation between the DNA binding, transactivation and repression activities of the deletion mutants is less clear than with the insertion mutants (Table 1). However, the results with the deletion mutants do not separate any protein regions uniquely involved in each activity. It is perhaps suprizing that there are mutants which, although unable to bind to DNA in our assay, are able to activate repression. It is possible that such mutants retain some DNA binding capability in the cell. These mutants are severely impaired in their ability to transactivate, and therefore demonstrate overlap between the protein domains

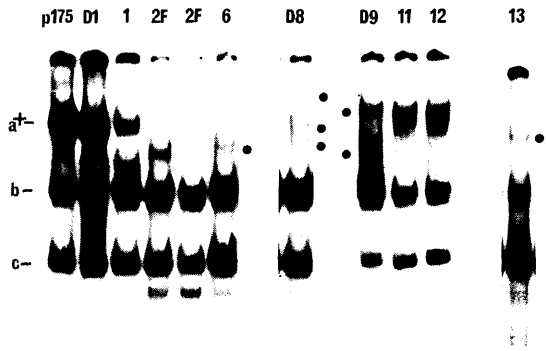

Figure 6: Binding assays performed at 0°C with antibody shift, using total cell extracts of cells transfected with deletion mutation plasmids pD1-15 (see Table 2). Only mutants with some detectable binding activity are shown. The wild type (p175) shifted complex (a^+) is labelled, as are the host derived complexes b and c; weak complexes are highlighted.

required for at least two of the three functions assayed.

Interestingly, deletions in regions 4 and 5 (pD9, D11 and D12) give rise to somewhat larger DNA complexes than that formed by wild type Vmw175, although these deletion plasmids are predicted to express proteins of a lower molecular weight. As suggested above for the insertion mutant pI29 this might be caused by a partial denaturation of these mutant polypeptides *in vivo* or *in vitro*. Some proteins deficient for nuclear localization (pD2F, D6 and D8) were able to bind to the IE3 probe, albeit very weakly, when extracts were prepared from whole cells (Figure 6); nuclear extracts of these deletion mutants did not contain significant amounts of Vmw175 (results not shown). It is also noteworthy that deletions pD1 and pD13 which remove a serine rich phosphorylation site in region 1 are also able to bind DNA.

DISCUSSION

In order to investigate the relationship between the functional activities of Vmw175 and its site-specific DNA binding property a panel of 39 in-frame insertion and sixteen deletion mutants were assayed for their ability to bind a DNA probe *in*

vitro. These mutants had previously been assayed for their ability to transactivate and repress herpes gene expression in transient assays (33). These studies had shown that two large regions, 2 and 4 in Figure 3, which are highly conserved between Vmw175 and the VZV "IE" protein 140K (28,29), do indeed correspond to important functional regions. Whilst region 2 seems to be of critical importance for both transactivation and repression functions, region 4 seems to be of lesser importance for repression.

Although the results presented in this paper do not demonstrate an absolute correlation between in vitro DNA binding activity and either transactivation or repression, it is clear that the general regions of the protein involved in all three phenotypes are similar. In particular the integrity of region 2 is of critical importance for each property investigated, excepting nuclear localization.

Previous studies have attempted to map the regions of Vmw175 involved in DNA binding by a variety of approaches. Kristie and Roizman (21) have reported that binding of monoclonal antibody H950 to Vmw175 abolishes DNA-binding activity. The epitope for this antibody has been mapped to within amino acids 21-36 (45) and therefore lies within a section of region 1 shown not to be required for DNA binding both by our results (Tables 1 and 2) and also by DeLuca and Schaffer (35). Therefore it is likely that the antibody is blocking DNA binding through longer range steric affects.

Hubenthal-Voss et al., (45) have also attempted to map the DNA binding domain of Vmw175 by using short synthetic oligopeptides to interfere or compete with functional sites for binding the recognition sites in the IE1 (α0) promoter. Their results, which mapped regions important for DNA binding activity near to the amino terminus, are at variance with our results. Indeed we show that the region they investigated seems to be of little importance, at least in binding the IE3 (α4) recognition site.

DeLuca and Schaffer (35) have used a set of viral mutants expressing truncated forms of Vmw175 to map the DNA binding

domain. The results obtained with their panel of deletion and nonsense mutants allowed them to present a map of the functional domains of the protein which is in good agreement with our own results presented here and previously (33). Mutants deleting the first 90 amino acids of the protein and those removing residues downstream from 773 were essentially wild type in their ability to bind an IE3 promoter DNA probe, and in their capacity to regulate IE3 and early gene transcription, although viruses encoding the downstream deletions were somewhat impaired in late gene expression. A mutant deleted from amino acid 590 was able to form a novel DNA complex at elevated salt concentrations, but possesed no regulatory actvity in the virus.

However, this approach lacks sensitivity unless applied from both the amino and carboxyl termini, and truncated proteins may exhibit conformational abnormalities making interpretation of results more complicated. Indeed from the results of our assays it is apparent that the insertion mutants give a more consistent pattern of results, and perhaps with the larger deletions only positive results should be considered significant. Our deletions pDl3, pDl and pD2F show that amino acids outside residues 229-496 are probably not directly involved in site-specific DNA binding. Those mutant polypeptides with insertions and deletions outwith this region which fail to bind DNA may be inactivated by longer range steric effects, for example disruption of protein folding.

Under the conditions of binding used by DeLuca and Schaffer (35) the Vmwl75 DNA complexes were salt labile when the concentration was raised from 15 to 65mM NaCl. Furthermore they observed that some of their mutants formed novel complexes at elevated salt concentrations. We did not observe this salt lability under our conditions of binding and indeed the wild type Vmwl75 DNA complex was stable up to 300mM NaCl (results not shown). Nor did we see any novel complexes formed at higher salt concentrations although these experiments were not performed on each mutant. We confirm their observations that deletion of the nuclear localization site, centred around amino acid 728 (33,35), did not prevent DNA binding of mutant protein recovered from the cytoplasm.

Michael et al (23) have recently reported that different post-translationally modified forms of Vmw175 possess different binding affinities for DNA probes which do not include an ATCGTC consensus. DeLuca and Schaffer (35) have shown that the serine rich tract in homology region A, which is conserved between VZV and HSV, is probably the major site of phosphorylation for this protein. However, we have shown that deletion of this region does not abolish affinity for the IE3 probe which includes the ATCGTC consensus, nor is this region essential for transactivation or repression (33). Interestingly, it has recently been reported that the serine phosphorylations of the adenovirus Ela protein do not affect transactivation or repression (46).

Because the regions of Vmw175 required for transcriptional activation and repression overlap with each other and with the region required for site-specific DNA binding we have not been able to directly associate site-specific binding uniquely with either regulatory activity. The weight of experimental evidence supports a role for the Vmw175 binding site at the cap site of IE gene 3 in autoregulation but it is still an enigma as to why the same protein sequences should apparently mediate this function and trancriptional activation which is not sequence specific. It is possible that Vmw175 possesses a second less specific DNA binding activity, perhaps encoded by the same protein sequences or alternatively elsewhere in the protein. Indeed Michael et al (23) have observed Vmw175 binding to the 5' transcribed non-coding regions of late genes which do not possess the consensus site-specific binding site. It will be interesting to investigate which regions of the protein are required for this activity, and whether these coincide with the regions (4 and 5) identified by DeLuca and Schaffer as being involved in late gene activation (35,47).

The precise functional role of regions 4 and 5 of Vmw175 is still obscure. We have previously shown that insertions and deletions in these regions interfere with transactivation and repression in a complicated manner (33) although clearly some mutants with large deletions in this region still encode proteins functional in transfection assays (34,48) and in recombinant

virus (35). Whether this portion of the coding sequence is simply important for conformational stability of the protein, or whether it directs a novel, uncharacterized activity is unknown. However, the strong conservation of region 4 between VZV and HSV, and the occurence of many temperature sensitive mutations in this region (13,47,49, unpublished results) suggests that this region does perform an essential role in the virus life-cycle.

Taking into consideration the known properties of Vmw175 it is possible to propose a simple functional model that could account for its dual roles of transcriptional activator and repressor. The model proposes that Vmw175 interacts simultaneously both with DNA and with host trancription factors, such as the TATA box binding factor TFIID (50,51), in order to produce a functional stabilized transcription complex. If the DNA binding region of Vmw175 encounters a very strong binding site sequence, such as that at the IE3 cap site, then the high affinity constant of the DNA/Vmw175 complex could effectively immobilize the transcriptional machinery. If the DNA sequence is less strongly bound by Vmw175, then the stabilized transcription complex would be free to proceed. Note that the proposed site of interaction with the host proteins may also map in region 2 which, at over 200 amino acids, is of itself almost as large as the adenovirus E1a protein. Thus the same region of the protein could be important for transactivation, repression and DNA binding. This model is consistent with the importance of the TATA/cap region for activation of the tk promoter (26) and the binding of Vmw175 to (non-consensus and possibly weak) sites in the 5' untranslated regions of other activated HSV genes (23). This model can be tested experimentally.

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REFERENCES

1. Honess, R.W. and Roizman, B. (1974) J. Virol. 14, 8-19.
2. Honess, R.W. and Roizman, B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1276-1280.
3. Clements, J.B., Watson, R.J. and Wilkie, N.M. (1977) Cell 12, 275-285.

4. Jones, P.C. and Roizman, B. (1979) J. Virol. 31, 299-314.
5. Everett, R.D. (1987) Anticancer Res. 7, 589-604.
6. Preston, C.M. (1979) J. Virol. 29, 275-284.
7. Watson, R.J. and Clements, J.B. (1980) Nature 285, 329-330.
8. Stow, N.D. and Stow, E.C. (1986) J. Gen. Virol. 67, 2571-2585.
9. Sacks, W.R. and Schaffer, P.A. (1987) J. Virol. 61, 829-839.
10. Sears, A.E., Halliburton, I.W., Meignier, B., Silver, S. and Roizman, B. (1985) J. Virol. 55, 338-346.
11. Sacks, W.R., Greene, C.C., Aschman, D.P. and Schaffer, P.A. (1985) J. Virol. 55, 796-805.
12. Longnecker, R. and Roizman, B. (1986) J. Virol. 58, 583-591.
13. Dixon, R.A.F. and Schaffer, P.A. (1980) J. Virol. 36, 189-203.
14. Pizer, L.I., Tedder, D.G., Betz, J.L., Wilcox, K.W. and Beard, P. (1986) J. Virol. 60, 950-959.
15. Beard, P., Faber, S., Wilcox, K.W. and Pizer, L.I. (1986) Proc. Natl. Acad. Sci. USA 83, 4016-4020.
16. Pereira, L., Wolff, M.H., Fenwick, M. and Roizman, B. (1977) Virology 77, 733-749.
17. Metzler, D.W. and Wilcox, K.W. (1985) J. Virol. 55, 329-337.
18. Hay, R.T. and Hay, J. (1980) Virology 104, 230-234.
19. Faber, S.W. and Wilcox, K.W. (1986) Nucl. Acids Res. 14, 6067-6083.
20. Kristie, T.M. and Roizman, B. (1986) Proc. Natl. Acad. Sci. USA 83, 3218-3222.
21. Kristie, T.M. and Roizman, B. (1986) Proc. Natl. Acad. Sci. USA 83, 4700-4704.
22. Muller, M.T. (1987) J. Virol. 61, 858-865.
23. Michael, N., Spector, D., Mavromara-Nazos, P., Kristie, T.M. and Roizman, B. (1988) Science 239, 1531-1534.
24. Everett, R.D. (1984) EMBO J. 3, 3135-3141.
25. Eisenberg, S.P., Coen, D.M. and McKnight, S.L. (1985) Mol. Cell. Biol. 6, 1940-1947.
26. Coen, D., Weinheimer, S.P. and McKnight, S.L. (1986) Science 234, 53-59.
27. Rixon, F.J., Campbell, M.E. and Clements, J.B. (1982) EMBO J. 1, 1273-1277.
28. McGeoch, D.J., Dolan, A., Donald, S. and Brauer, D.H.K. (1986) Nucl. Acids Res. 14, 1727-1764.
29. Davison, A.J. and McGeoch, D.J. (1986) J. Gen. Virol. 67, 597-611.
30. Everett, R.D. and Dunlop, M. (1984) Nucl. Acids Res. 12, 5969-5978.
31. Felser, J.M., Straus, S.E. and Ostrove, J.M. (1987) J. Virol. 61, 225-228.
32. Felser, J.M., Kinchington, P.R., Inchauspe, G., Srauss, S.E. and Ostrove, J.M. (1988) J.Virol. 62, 2076-2082.
33. Paterson, T. and Everett, R.D. (1988) Virology 166, 186-196.
34. DeLuca, N.A. and Schaffer, P.A. (1987) Nucl. Acids Res. 15, 4491-4511.
35. DeLuca, N.A. and Schaffer, P.A. (1988) J.Virol. 62, 732-743.
36. Everett, R.D. (1987) EMBO J. 6, 2069-2076.
37. Everett, R.D. (1986) J. Gen. Virol. 67, 2507-2513.
38. Brown, S.M., Ritchie, D.A. and Subak-Sharpe, J.H. (1973) J. Gen. Virol. 68, 1-18.

39. Corsalo, C.M. and Pearson, M.L. (1981) Somatic cell genetics 7, 603-616.
40. Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745-2752.
41. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucl. Acids Res. 11, 1475-1489.
42. Showalter, L.D., Zweig, M. and Hampar, B. (1981) Infect. Immun. 34, 684-692.
43. Garner, M.M. and Revzin, A. (1981) Nucl. Acids Res. 9, 3047-3060.
44. Russell, J., Stow, E.C., Stow, N.D. and Preston, C.M. (1987) J. Gen. Virol. 68, 2397-2406.
45. Hubenthal-Voss, J., Houghten, R.A., Pereira, L. and Roizman, B. (1988) J. Virol. 62, 454-462.
46. Richter, J.D., Slavicek, J.M., Schneider, J.F. and Jones, N.C. (1988) J. Virol. 62, 1948-1955.
47. DeLuca, N.A., Courtney, M.A. and Schaffer, P.A. (1984) J. Virol. 52, 767-776.
48. Mavromara-Nazos, P., Silver, S., Hubenthal-Voss, J., McKnight, J.C. and Roizman, B. (1986) Virology 149, 152-164.
49. Preston, V.G. (1981) J. Virol. 39, 150-161.
50. Sawadogo, M. and Roeder, R.G. (1985) Proc. Natl. Acad. Sci. USA 82, 4394-4398.
51. Sawadogo, M. and Roeder, R.G. (1985) Cell 43, 165-175.