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THE CONTROL OF HERPESVIRUS IMMEDIATE EARLY GENE EXPRESSION

by

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A Thesis presented for the degree of
Doctor of Philosophy

in

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RESULTS AND DISCUSSION

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SUMMARY

The experiments performed in this study investigate the regulation of the immediate early (IE) genes of herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV).

Transcription mediated by an HSV-1 IE gene promoter and upstream regulatory sequences is stimulated by a structural virion component (Post *et al.*, 1981). In order to identify the *trans*-inducing factor (TIF) involved, a series of HSV-1 genomic clones were transfected into baby hamster kidney (BHK) cells together with chimaeric plasmids which contained the thymidine kinase (TK) gene under IE control. Fragments, EcoRI i (0.62 to 0.72 map units), EcoRI b (0.72 to 0.87map units) and BamHI f (0.64 to 0.69 map units), a sub fragment of EcoRI i, were found to elevate TK expression in this assay. The stimulatory sequences were localised to to a 2.6kb fragment, contained within the plasmid, pMC1. According to the mapping data of Hall *et al.* (1981) this region contained the complete sequences only of a 1.7kb transcript. The stimulatory effect was abolished by an 8bp linker insertion, which disrupted the reading frame of this gene, but not by other insertions within BamHI f. Induction mediated by the BamHI f fragment was confined to genes under the control of IE sequences, showing it to have the same specificity as the virion TIF, whereas EcoRI b mediated a general stimulatory effect. These experiments indicated that the 1.7kb gene within BamHI f encoded the TIF and that polypeptides encoded by EcoRI b were unlikely to be involved.

Hybridisation of pMC6 (a subclone of pMC1) to infected cell RNA, prior to its translation *in vitro*, prevented the synthesis of a polypeptide of molecular weight 65,000. Immunoprecipitation of translated samples with monoclonal antibody identified this species as Vmw65, a major structural polypeptide. The gene encoding this polypeptide was found to possess an efficient promoter which could direct efficient expression in the absence of viral *trans*-activating polypeptides. The studies performed

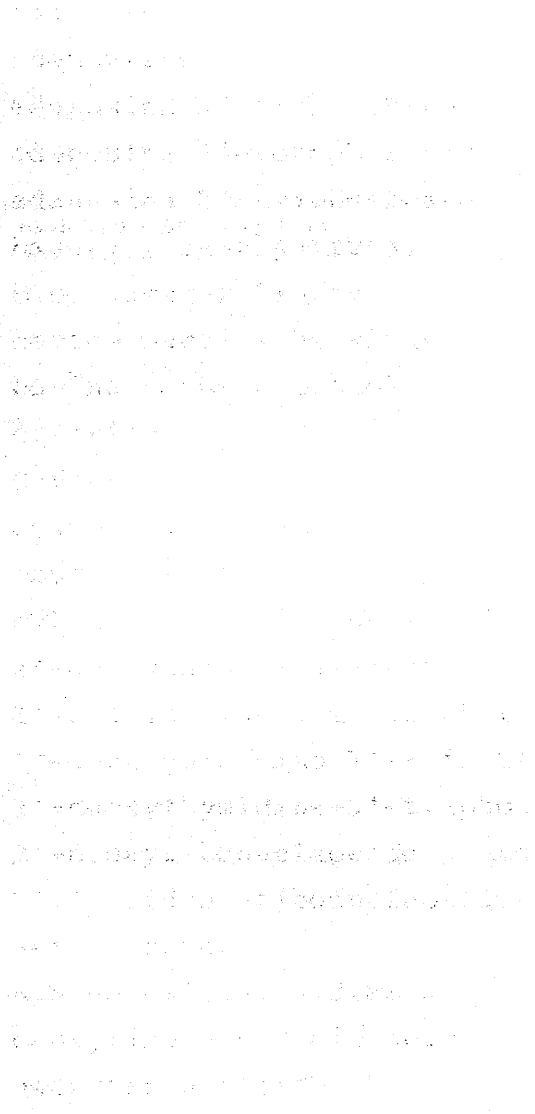
identify the virion factor responsible for trans-induction of IE genes as Vmw65, a major structural polypeptide, located in the tegument of the virion.

Expression of HSV-1 regulated genes is not increased by infection with PRV, a related herpesvirus (Batterson and Roizman, 1983). Experiments were therefore carried out to examine the control of the PRV major IE gene. The 5' terminus of the mRNA was mapped by S1 nuclease analysis and hybrid plasmids, which contained IE upstream sequences linked to the HSV-1 TK gene, were constructed. Gene expression under the control of PRV IE or HSV-1 IE gene 3 upstream regions was compared using transient assays. It was found that infection with UV-irradiated PRV did not stimulate expression from PRV IE or from HSV-1 IE gene 3 upstream regions in BHK or pig kidney cells, indicating that PRV did not possess an effective TIF. Infection with UV-treated HSV-1, or cotransfection with pMC1 (which encodes Vmw65) stimulated expression from both PRV and HSV IE gene upstream regions. However, co-infection with PRV and HSV-1 did not result in increased synthesis of the PRV IE polypeptide.

The nucleotide sequence of the 5' end of the PRV transcript and its upstream region was determined. This region was unlike the upstream regions of HSV IE genes in overall structure, but showed a strong similarity to the enhancers of human and murine cytomegaloviruses (HCMV and MCMV). In particular, a reiterated 15bp element of the PRV upstream region was homologous to a conserved, repeated sequence element found in both HCMV and MCMV enhancer regions, and was also related to the "TAATGARATTC" motif found upstream of all HSV IE genes.

In order to facilitate future investigations concerning the mechanism of action of the HSV-1 TIF, Vmw65, preliminary purification and characterisation were carried out. A polypeptide of molecular weight, 65,500 was partially purified from HSV-1 virions by treatment with NP40. Immunoprecipitation studies identified this species as the TIF, and also showed that a 65,000 molecular weight binding protein isolated from infected cells was unrelated.

Vmw65 has no affinity for double-stranded calf thymus DNA, suggesting that it does not mediate transcriptional regulation by direct interaction with IE upstream sequences.



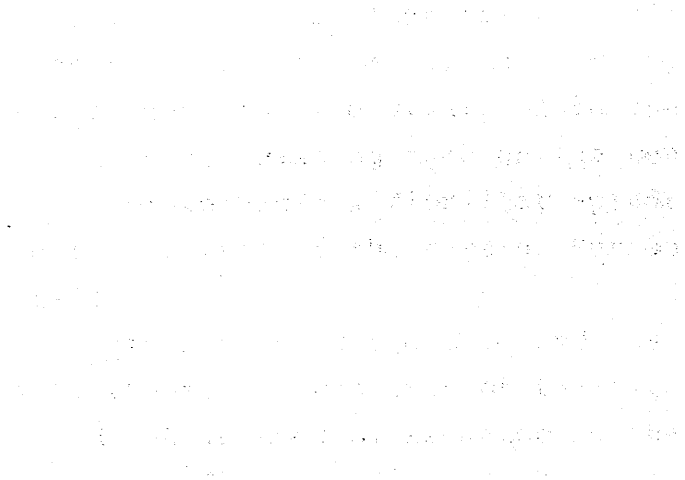
ABBREVIATIONS

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

A	adenine
aa	amino acid
Ad	adenovirus
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
APS	ammonium persulphate
b, bp	base(s), base pairs(s)
BHK	baby hamster kidney
BMV	bovine mamillitus virus
BSA	bovine serum albumin
C	cytosine
Ci	Curie(s)
CPE	cytopathic effect
cpm	counts per minute
CTP	cytidine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphosphate
ddNTP	2',3'-dideoxyribonucleoside-5'-triphosphate
DATD	N,N'-diallytartardiamide
DNA	deoxyribose nucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
EBV	Epstein-Barr virus
E. coli	Escherichia coli
EDTA	sodium ethylenediamine tetra-acetic acid
EGTA	ethylene glycol-bis(β -amino-ethyl ether)N,N,N',N'-tetra-acetic acid.
EHV	equid herpes virus

G	guanine
GTP	guanosine-5'-triphosphate
h	hour(s)
HCMV	human cytomegalovirus
HeBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
HSV	herpes simplex virus
kb, kbp	kilobase(s), kilobase pair(s)
LTR	long terminal repeat
MCMV	murine cytomegalovirus
min	minute(s)
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
mu	map units
mw	molecular weight
N	unspecified nucleotide (A, G, C or T)
NP40	nonidet P40
NPT	non-permissive temperature
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming units
PIPES	piperazine-N,N'-bis(2-ethane sulphonic acid)
PK	pig kidney
PRV	pseudorabies virus
R	purine
RNA	ribonucleic acid
RNAase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SV40	simian virus 40
T	thymine
TEMED	N,N,N',N'-tetramethylethylene diamine
TK	thymidine kinase
tris	tris(hydroxymethyl)aminomethane

tRNA	transfer ribonucleic acid
ts	temperature-sensitive
UV	ultra-violet
v/v	volume/volume
Vmw	molecular weight of viral polypeptide in kilodaltons
VP	virion protein
VZV	varicella zoster virus
wt	wild type
w/v	weight/volume
Y	pyrimidine



INTRODUCTION

Members of the family Herpesviridae are characterised by a double-stranded, linear DNA genome in the core of the virion, a capsid consisting of 162 capsomeres, which is assembled in the cell nucleus, and an envelope derived from the nuclear membrane.

To date more than 80 species of herpesviruses have been identified. Of these, six have been isolated from humans, and the remainder from a wide variety of higher eukaryotic hosts (Roizman and Batterson, 1985).

In this introduction, attention is focussed on the molecular biology of two species of herpesvirus, herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV), although attempts have been made to discuss relevant information concerning other herpesviruses. A brief review of eukaryotic transcription, pertinent to the results presented in this thesis, is also included.

1 THE HERPESVIRUSES

1.1 The herpes virion

The herpesvirion has four distinct morphological elements: core; capsid; tegument; and envelope.

The core contains the viral DNA in the form of a ring-like structure. Passing through the centre of this structure is a proteinaceous fibrillar spindle, thought to be embedded in the wall of the capsid (Furlong et al., 1972; Nazerian, 1974).

The capsid surrounds the core and has a diameter of approximately 100nm. It consists of 162 capsomeres (12 pentameres and 150 hexameres) arranged in the form of an icosadeltahedron. Electron microscopy studies have shown the hexameric subunits to be 9.5 x 12.5nm in longitudinal cross-section, with a 4-nm channel running through the axis (Wildy et al., 1960). The tegument is defined as the structure located between capsid and envelope (Roizman and Furlong, 1974) and may be fibrous in appearance (Morgan et al., 1968; Schwartz and Roizman, 1969). The thickness of

this layer varies considerably among the herpesvirus (Nazerian and Witter, 1970; McCombs et al., 1971).

The envelope is the outermost structure of the virion and consists of a trilaminar membrane (Epstein, 1962) which appears to be derived from sections of altered nuclear membrane (Asher et al., 1969; Morgan et al., 1959). Numerous spikes, estimated to be approximately 8nm in length, protrude through the envelope (Wildy and Watson, 1963).

1.2 Biology of herpesviruses

1.2.1 Classification

Herpesviruses vary greatly in terms of their biological properties but a ubiquitous characteristic of this family is the ability to maintain a latent infection in their host. The members of the Herpesviridae have been classified into three subfamilies, alpha, beta and gamma herpesvirinae on the basis of four biological characteristics: host range, rate of multiplication, cytopathology and characteristics of latent infection (Matthews, 1982; Roizman, 1982).

Alphaherpesvirinae have a short reproductive cycle. Infection spreads rapidly in culture, destroying infected cells and a latent infection is frequently established in the ganglia.

Betaherpesvirinae have a restricted host range, long replicative cycle and the spread of infection in cell culture proceeds slowly, causing enlargement of infected cells. Latently infected tissue includes secretory glands, lymphoreticular tissues and kidney cells.

Gammaherpesvirinae are usually restricted to the family of the natural host. Viruses of this group are specific to T or B lymophocytes in which infection is frequently arrested.

Classification of some of the best studied herpesviruses is shown in Table 1.

Table 1. Sub family and genomic type of selected herpesviruses.

Host	Common name	Sub family	Genomic type	Molecular weight (mega daltons)
human	herpes simplex virus (type 1)	alpha	E	96
human	herpes simplex virus (type 2)	alpha	E	96
human	* varicella-zoster virus	alpha	E	100
human	Epstein-Barr virus	gamma	C	114
human	cytomegalovirus	beta	E	145
monkey	herpesvirus saimiri	gamma	B	103
mouse	murine cytomegalovirus	beta	F	155
cow	bovine mamillitis virus	alpha	E	88
pig	pseudorabies virus	alpha	D	91
chicken	Marek's disease virus	gamma	E	110
catfish	channel catfish virus	alpha	A	86

* The U_L segment of VZV is flanked by a short inverted repeat of 88.5bp which allows limited inversion of the L segment (+Davison, 1984).

References for the genome structures are as follows: HSV-1, Sheldrick and Berthelot (1974); HSV-2, +Cortini and Wilkie (1978); VZV, +Davison (1984); EBV, +Given and Kieff (1978); HCMV, +LaFemina and Hayward (1980); herpesvirus saimiri, +Fleckenstein and Borkhamm (1975); MCMV, Ebeling et al. (1983), bovine mamillitis virus, +Ludwig (1982); PRV, Ben-Porat et al. (1979); Marek's disease virus, +Cebrian et al. (1982); channel catfish virus, +Chousterman et al. (1979).

+ Listed at the end of the reference section.

1.2.2 Pathogenicity

1.2.2.1 Diseases caused by human herpesviruses

Herpes simplex virus type 1 is responsible for vesicular lesions of the lips and nasal membranes, ocular keratitis and occasionally more severe symptoms, including encephalitis (Smith et al., 1941; Gallardo, 1943; Rawls, 1985). HSV-1 is maintained in a latent state in the trigeminal ganglia and may be periodically reactivated to produce recurrent symptoms (Baringer et al., 1974).

Herpes simplex virus type 2 is a sexually transmitted disease which produces lesions in the genital regions. This virus remains latent in the sacral ganglia with occasional outbreaks of lytic infection (Klein, 1982; Knox et al., 1982).

Varicella-zoster virus is responsible for chickenpox and shingles. Chickenpox is a childhood disease, while shingles, a localised vesicular condition occurs in adults and is presumed to be caused by reactivation of latent VZV (Weller et al., 1958, Gelb, 1985).

Human cytomegalovirus usually infects salivary glands but may also be found in other tissues, usually resulting in a mild or subclinical disease although congenital neonatal infection is sometimes associated with severe neuronal damage (Alford and Britt, 1985). Organ transplants or blood transfusions in immunosuppressed individuals frequently result in outbreaks of HCMV infection (Ho, 1982).

Epstein-Barr virus is the causative agent of infectious mononucleosis and is involved in the aetiology of Burkitt's lymphoma, neopharyngeal carcinoma and lymphomas of immunosuppressed individuals (Neiderman et al 1976; Miller, 1985).

Human B-lymphotropic virus has recently been isolated from immunocompromised patients (Salahuddin et al., 1986).

1.2.2.2 Examples of non-human herpesvirus diseases

Pseudorabies virus results in a natural infection in swine similar to HSV in man, producing only a mild infection in adult pigs but a frequently terminal illness in new born animals and is also responsible for Aujezky's disease, a fatal condition in cattle (McKercher, 1973; Gustafson,

1981). Pseudorabies virus persists in a latent state in infected pigs, a crucial factor in the spread of the virus to susceptible animals (Beran et al., 1980).
Equid herpesvirus 1 causes pregnant mares to abort.
Marek's disease virus causes a highly contagious neurolymphomatosis in chickens.
Simian herpesviruses, HVS and HVA produce malignant lymphomas in certain primate hosts (Melendez et al., 1969, 1972).

1.3 Genome structure

1.3.1 Structure of the genomes of herpesviruses

The DNA of herpesviruses is linear and double-stranded as shown by electron microscopy studies (Becker et al., 1968) and has a molecular weight in the range of 80 to 150×10^6 (Roizman and Furlong, 1974).

The percentage of G+C residues varies markedly among the herpesviruses. PRV has a high G+C content of 73%, HSV-1 68% and VZV 46% (Ben-Porat and Kaplan, 1962; Kieff et al., 1971). Herpesvirus DNAs fragment on denaturation with alkali (Kieff et al., 1971; Wilkie, 1973) and the lability of the DNA is considered to be the result of single stranded nicks at random sites in the genome (Ben Porat et al., 1979). This feature may be partly attributable to the incorporation of ribonucleotides in the packaged DNA (Biswal et al., 1974).

Probably the most interesting feature of herpes virus genomes is their pattern of reiterations, and it is possible to group the herpesviruses according to their genome structures (Roizman, 1982). It should, however, be noted that only a minority of species have been analysed in sufficient detail to permit such a classification. A diagram of each form of genome arrangement is shown in figure 1. Groups A to E were originally described by Roizman (1982), and the genome arrangement of MCMV (Ebeling et al., 1983) has been designated group F. Examples of species possessing each type of genome arrangement are shown in table 1. It should be noted that grouping according to structural criteria does not correlate with the biological

VIRUS	NO OF ISOMERS	GENOME TYPE
CCV	1	A
HVS	1	B
EBV	1	C
PRV	2	D
HSV	4	E
MCMV	1	F

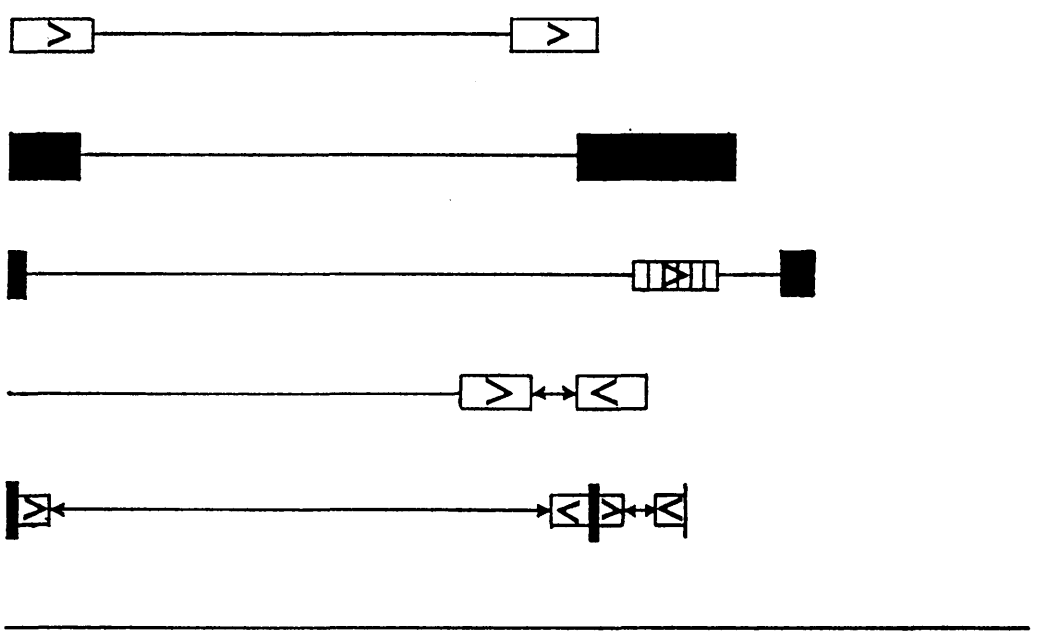


Figure 1. Sequence arrangements of herpesvirus genomes. Genome types A-F are exemplified by channel catfish virus (CCV), herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), pseudorabies virus (PRV), herpes simplex virus (HSV), and murine cytomegalovirus (MCMV). Horizontal lines represent unique sequence. Reiterated sequences of greater than 1kb are shown by open rectangles, and the arrowheads within the rectangles indicate whether repeats are direct or inverted. Solid rectangles denote terminal reiterations which are directly repeated. In genome type E the terminal reiterations ('a' sequences) are also reresented internally in the inverted orientation. The number of isomers is indicated and possible invertions of unique sequence are denoted by arrows.

classification.

1.3.2. Analysis of genome arrangement of HSV and PRV

Studies of HSV DNA by electron microscopy have shown structures consistent with a model of the genome which consists of two unique regions (U_L and U_S) bounded by inverted repeats (Sheldrick and Berthelot, 1974). Further research revealed that the repetitions (R_L and R_S) bounding U_L and U_S are distinct and that inversions of the unique regions occur. This phenomenon results in the occurrence of four isomers (Hayward et al., 1975; Delius and Clements, 1976; Clements et al., 1976). In addition, HSV-1 DNA has terminal direct repeats, as shown by the fact that renaturation after digestion of the termini with a 5' to 3' exonuclease results in a circular form (Wadsworth et al., 1975). This repeat termed the 'a' sequence, is composed of reiterated elements interspersed with unique sequence and has been found to vary by between 280 and 550bp depending on the virus strain. The difference appears to be predominantly due to variable copy numbers of the reiterated units (Davison and Wilkie, 1981; Mocarski and Roizman, 1982). The 'a' sequence of HSV-1 strain 17 has the format: $(DR1)(U_a)(DR2)_{18}(U_b)(DR1)$, where U_a and U_b are unique sequences and DR1 and DR2 are direct repeats of 17 and 12bp, respectively (Davison and Wilkie, 1981). Families of multiple copies of short directly repeating sequences, with unit lengths of up to 54 residues have also been identified in each of the genome segments (Rixon et al., 1984; McGeoch et al., 1985; Perry, 1986).

Cis-acting sequences for the inversion of L and S components of the HSV-1 genome relative to each other are found to be contained within the 'a' sequence (Mocarski et al., 1980; Mocarski and Roizman, 1982; Chou and Roizman, 1985). However, isomerisation of the genome of some HSV-1 mutants which lack 'a' sequences is found to occur (Pogue-Geile et al., 1985; Longnecker and Roizman, 1986), indicating that repeat regions may, in some instances, substitute for 'a' sequences, allowing intermolecular and intramolecular recombination to occur. The HSV-1 'a'

sequence is also involved in cleavage and packaging of the viral genome (Davison and Wilkie, 1981; Mocarski and Roizman, 1982; section 2.4.3).

PRV has only one set of inverted repeats which border the short unique region (Stevely, 1977) and does not possess terminal direct repeats analogous to the HSV-1 'a' sequence (Ben-Porat and Veach; 1980). However, two isomeric forms of the genome are apparent, both of which are infectious (Ben-Porat et al., 1980). It has been proposed that recombination of the parent DNA molecules prior to replication results in the inversion of the genome (Ben-Porat et al.,1982).

1.3.3. Sequence homologies between herpesviruses

Renaturation kinetics and Southern blot analysis has shown sequence homologies to exist between members within each of the herpesvirus sub-families. For example, the alphaherpesviruses, HSV-1, HSV-2, PRV, VZV, EHV-1 and BMV all show detectable levels of cross hybridisation. The homology between HSV-1 and HSV-2 is estimated at 50% (Kieff et al., 1972) and between HSV-1 and PRV, at 8% (Ludwig et al., 1972; Bronson et al., 1972).

The nucleotide sequence of EBV, VZV, HSV-1 and the short region of HCMV has been determined (Baer et al.,1984; Davison and Scott, 1986; McGeoch et al.,1985; Weston and Barrell, 1986; D.J. McGeoch, personal communication), allowing the evolutionary relationships between these viruses to be assessed. Seven genes from the short region of VZV were found to share homology with those from the short region of HSV-1, but the arrangement and location of the genes was found to differ (Davison and Wilkie, 1983b; Davison and Scott, 1985; McGeoch et al., 1985). Epstein-Barr virus (a gammaherpesvirus) has no equivalent of the short region of the alphaherpesviruses and the short segment of HCMV (a betaherpesvirus) does not share significant homology with sequences from HSV-1 or PRV (Weston and Barrell, 1986). The U_L regions of VZV and HSV-1 are largely colinear. However, Southern blot analysis has shown that a segment of approximately 40kb is inverted in

PRV with respect to HSV-1 (Ben-Porat et al., 1983b; Davison and Wilkie, 1983b) (figure 2).

Approximately 30 of the VZV genes have detectable homologues in EBV, although the two viruses have extensively diverged and large scale sequence rearrangements have occurred (Davison and Taylor, 1987).

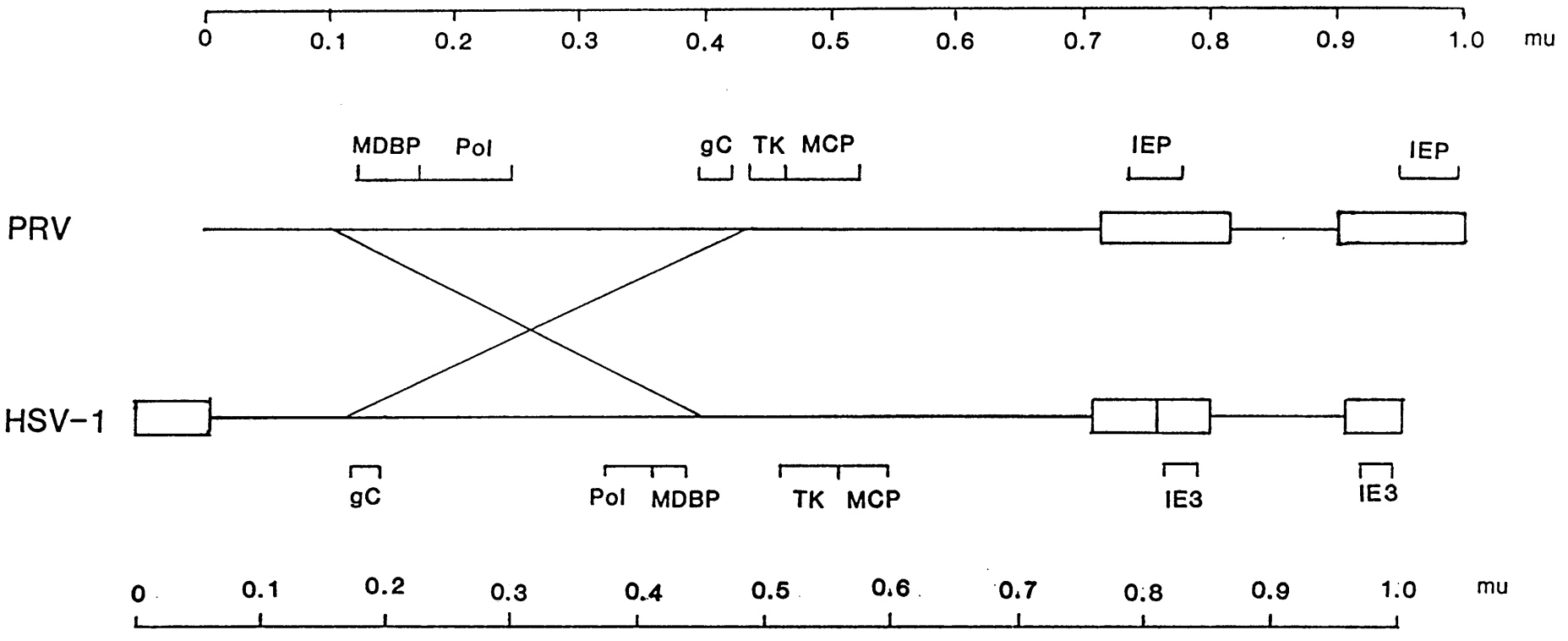

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
mu
MDBP
Pol
gC
TK
MCP
IEP
IEP
PRV
HSV-1
gC
Pol
MDBP
TK
MCP
IE3
IE3
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
mu

Figure 2. Comparisons of genes encoding known functions on the HSV-1 and PRV genomes.

The genes indicated also represent regions of high cross homology between the two genomes (Ben-Porat et al., 1983b; Davison and Wilkie, 1983b). The crossed lines denote the region of inversion in PRV with respect to HSV-1. The genome of HSV-1 is shown with U_L in the inverted orientation with respect to the prototype arrangement (figure 4).

2 REPLICATION OF HERPESVIRUSES

Herpes simplex virus type 1 is the most extensively characterised herpesvirus and studies concerning the replication of HSV-1 have provided a model for other members of the group. The following sections of the introduction will therefore concentrate on research carried out on this virus.

2.1 Initial stages of infection

Herpes virions penetrate the cell by fusion of the envelope with the cell plasma membrane (Morgan et al., 1968). Mutants tsB5 and tsJ12, if grown at NPT, produce defective glycoprotein B (gB). The mutant virions are capable of binding, but are unable to penetrate the cell, showing gB to be required for this process (Sarmiento et al., 1979; Little et al., 1981). Upon entry, the capsid is transported to the nuclear pores where the DNA is released into the nucleoplasm. A viral function is necessary, as capsids of the mutant tsB7 accumulate at the nuclear pores and release viral DNA only after downshift to the permissive temperature (Batterson et al., 1983; Knipe et al., 1981).

2.2 Gene expression in HSV-1

2.2.1 Regulation of transcription

The replicative cycle of the HSV genome is temporally controlled and gene expression, as studied by examination of the kinetics of specific polypeptides or the transcripts encoding them, can be divided into three general phases termed immediate early (IE), early and late (Clements et al., 1977) or α, β and γ (Honess and Roizman, 1974). IE genes are transcribed directly after release of viral DNA into the nucleus and have no requirement for de novo protein synthesis. They are therefore transcribed efficiently in the presence of translational inhibitors such as cycloheximide (Kozak and Roizman, 1974; Swanstrom and Wagner, 1974; Clements et al., 1977; Jones and Roizman, 1979; Harris-Hamilton and Bachenheimer, 1985). As discussed later

(section 2.2.5) a component of the virus particle strongly stimulates the expression of HSV IE genes (Post et al., 1981; Batterson and Roizman, 1983). Immediate early polypeptides have been implicated as trans-activators of later stages of gene expression. It has been shown conclusively that Vmw175, an IE gene product is an essential control factor in the transition from IE to early and late stages of gene expression.

The regulatory role of Vmw175 was elucidated using mutants with ts defects in this polypeptide. These mutants fail to transcribe early and late genes at NPT (Dixon and Schaffer, 1980; Preston, 1979a). This pattern of transcription was also observed for mutants carrying extensive deletions in Vmw175 (DeLuca et al., 1985). Functional Vmw175 is continuously required throughout the replicative cycle to prevent the reversion to the IE pattern of transcription (Watson and Clements, 1978, 1980; Preston, 1979a).

Early genes are abundantly expressed in the absence of DNA replication (Swanstrom et al., 1975). A number of early genes are known to encode enzymes required for the synthesis of viral DNA.

Two subclasses of late mRNA γ_1 and γ_2 are readily distinguishable. In the absence of DNA replication γ_1 mRNAs are present whereas no (or only very low amounts of) γ_2 mRNA can be detected. Both subclasses, however, require DNA synthesis to attain their maximum rate of transcription (Jones and Roizman, 1979; Holland et al., 1980; Gibson and Spear, 1983; Johnson et al., 1986). Many of the late proteins identified appear to be constituents of the virion.

There is evidence to suggest that HSV transcription is negatively as well as positively regulated. Godowski and Knipe (1983, 1985, 1986) proposed that the major DNA binding protein (MDBP), an early species is involved in the repression of expression at all stages of infection, as elevated levels of transcriptional activity were observed during infection at NPT with a mutant exhibiting a ts defect in the MDBP. The IE polypeptide, Vmw175, has been implicated in an autoregulatory function, based on the the

fact that mutants with a ts lesion in this polypeptide synthesise increased amounts of IE mRNAs and polypeptides. (Preston, 1979a and b; Dixon and Schaffer, 1980; Watson and Clements, 1980).

Immediate early, but not early and late transcripts are thought to be subjected to post transcriptional control which blocks the synthesis of IE polypeptides (Kozak and Roizman, 1974; Preston, 1979a; Harris-Hamilton and Bachenheimer, 1985).

2.2.2 HSV transcripts

The mRNAS of HSV share a number of properties with those of the host cell. Both are transcribed in the nucleus by RNA polymerase II (Ben-Zeev and Becker, 1977; Costanzo et al., 1977), are 3' polyadenylated and capped at the 5' terminal and are internally methylated (Bachenheimer and Roizman, 1972; Silverstein et al., 1973; Bartoski and Roizman, 1976; Moss et al., 1977)

Synthesis of HSV mRNAs is directed by a promoter located upstream of each gene. The 5' regions of HSV genes possess regulatory elements analogous to those found in other eukaryotic promoters, enabling their recognition by the cellular transcription machinery. HSV transcripts are found to possess the polyadenylation signal AATAAA, or near variant (Wagner, 1985), which is also recognised as a feature of eukaryotic transcripts (Proudfoot and Brownlee, 1976) and 3' flanking sequences contain the sequence YGTGTTYY, which is believed to be necessary for efficient termination (McLauchlan et al., 1985).

The high degree of splicing observed in many cellular and viral systems is not found in HSV, as only a minority of HSV genes contain introns (Frink et al., 1981, 1983; Watson et al., 1981; Costa et al., 1985). A large number of HSV transcripts are partially overlapping and arranged in nested groups. These transcripts frequently have common 3' ends but distinct 5' termini, for example, US10, 11 and 12 are arranged in this way. The US11 and US12 transcripts are also found to share out of phase coding sequences (Rixon and McGeoch, 1984). Families of genes with common 5'ends and

different 3'ends are also found and are the result of read through of polyadenylation sites (Anderson et al., 1981; Hall et al., 1982).

The nucleotide sequence of the HSV-1 genome (strain 17) has now been determined. Analysis of open reading frames within the sequence plus S1 nuclease mapping data has provided an extensive transcript map (Wagner, 1984, 1985; Rixon and McGeoch, 1985; Costa et al., 1985; Dalrymple et al., 1985; McGeoch et al., 1985). U_S is 12,979kb in length and contains twelve protein coding regions (US1-12) as depicted in figure 3 (McGeoch et al., 1985; Rixon and McGeoch, 1985), TR_S is 6,677kb and encodes 1 gene (McGeoch et al., 1986) and TR_L is 9,214kb and is thought to contain 2 genes (Perry, 1986; Chou and Roizman, 1986). U_L is 107,994kb and contains 57 large open reading frames (D.J. McGeoch, personal communication) but detailed mapping data is not yet available for the entire region. The total size of the HSV-1 genome is therefore 152,260kb and it is estimated to encode 74 genes, three of which are present in two copies. However, the exact size of the genome is expected to vary due to differing copy numbers of reiterated units which occur in each of the segments.

A number of enzymes and structural and regulatory polypeptides have been mapped and characterised by the study of mutants (Stow et al., 1978, Preston et al., 1979b; Conley et al., 1981; Knipe et al., 1981). Alternatively, expression of cloned genes encoding an assayable function in heterologous systems has permitted identification and study of the protein product (Preston and Cordingley, 1982; Everett, 1984b; Campbell et al., 1984; Preston and Fisher, 1984). However, as yet, the function of a large number of HSV-1 gene products remains unknown. The map locations of genes encoding known functions are shown in figure 4.

2.2.3 Regulation of expression and mapping of PRV genes

Gene expression in PRV, like that of HSV, occurs in IE, early and late phases. These groups have kinetics and requirement for protein synthesis similar to equivalent classes in HSV (Rakusanova et al., 1971; Jean et al., 1974;

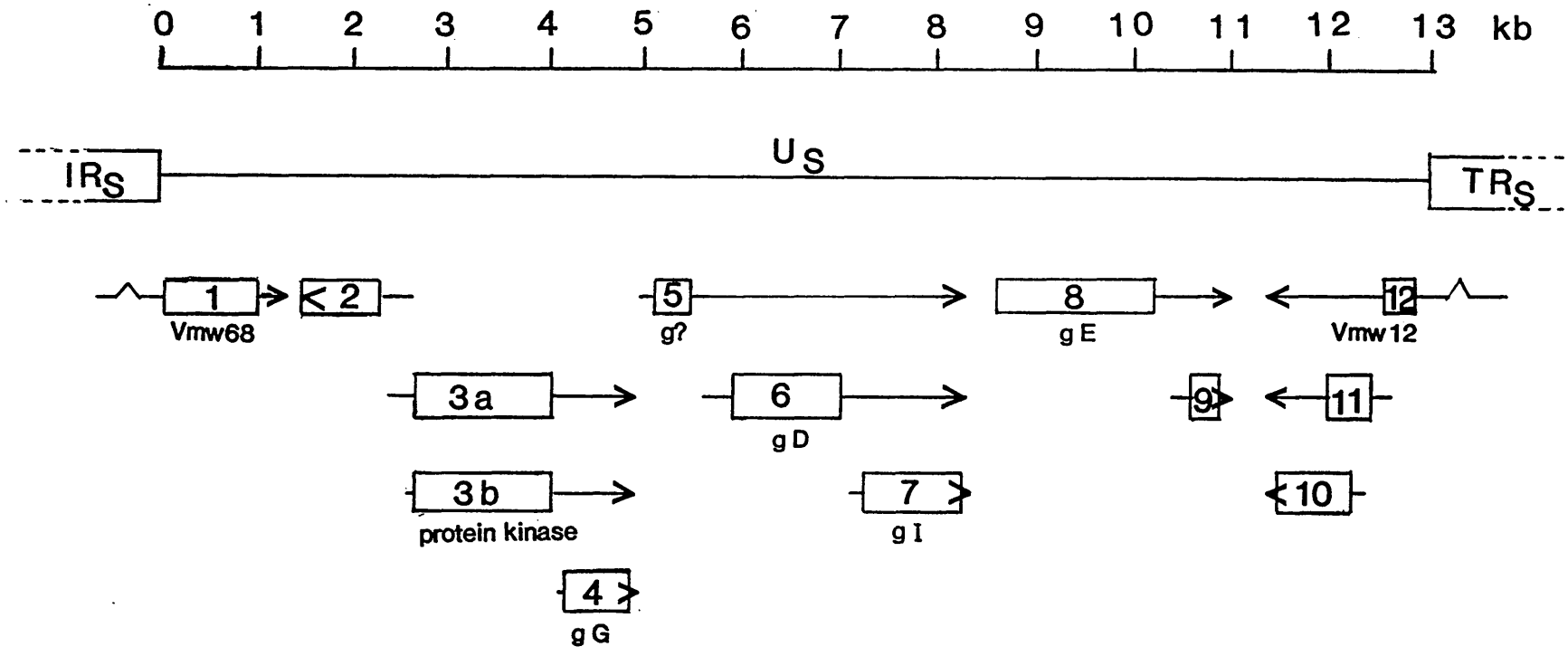

0 1 2 3 4 5 6 7 8 9 10 11 12 13 kb
IRS
US
TRS
1
Vmw68
2
5
g?
8
gE
12
Vmw12
3a
6
gD
9
11
3b
protein kinase
7
gI
10
4
gG

Figure 3. The mRNAs mapping within U_S.

The mRNA species are numbered 1-12 as described by McGeoch et al. (1985) and Rixon and McGeoch (1985). The untranslated portion of each mRNA is represented by a line and the probable polypeptide coding region is boxed. The direction of transcription is indictated by an arrowhead. Known polypeptide products are shown below mRNA mapping position. g? indicates a putative glycoprotein, predicted from sequence data (McGeoch, 1985).

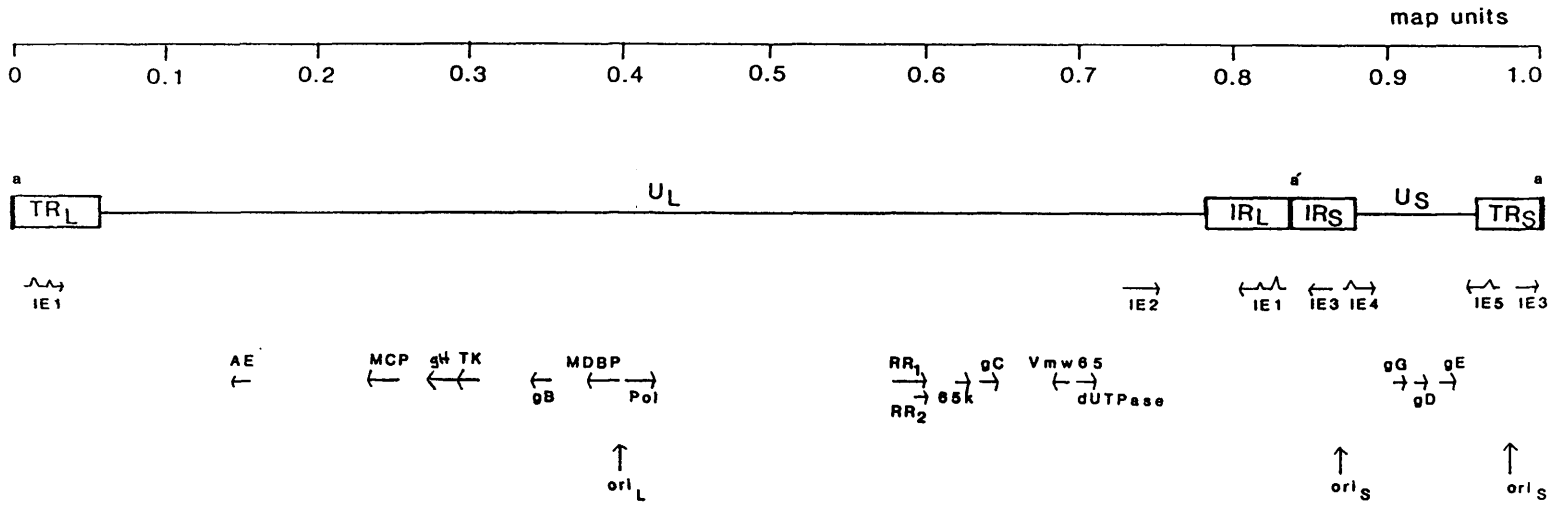

map units
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
a
TRL
UL
a'
IRL
IRS
US
a
TRS
IE1
IE2
IE1
IE3
IE4
IE5
IE3
AE
MCP
gH
TK
MDBP
gB
Pol
RR1
RR2
65k
gC
Vmw65
dUTPase
gG
gD
gE
orIL
orIS
orIS

Figure 4. Organisation of the HSV-1 genome.

The HSV-1 genome is shown in the prototype orientation. Unique regions (U_L) and (U_S) are flanked by terminal (TR) and internal repeats (IR) depicted by open rectangles and the 'a' sequences are indicated. Below the genome the map positions of IE mRNAs and the early and late mRNAs which specify the best characterised polypeptides and the origins of replications are shown. Abbreviations and references for mapping data are as follows:

IE immediate early, IE1, Perry et al. (1986), IE2, Mackem and Roizman (1982b), IE3, Rixon et al. (1982), IE4 and IE5, Rixon and Clements (1982).

AE alkaline exonuclease, Costa et al. (1983).

MCP major capsid protein, Costa et al. (1983).

TK thymidine kinase, McKnight (1980).

gB glycoprotein B, Bzik et al. (1984).

MDBP major DNA binding protein, Rafield and Knipe (1984).

Pol DNA polymerase, Quinn and McGeoch (1985).

RR ribonucleotide reductase, McLauchlan and Clements (1983).

65K DNA binding protein (mw 65,000), Marsden et al(1987).

Vmw65 trans-inducing factor (mw 65,000), Dalrymple et al. (1985).

gC glycoprotein C, Frink et al. (1983).

dUTPase Preston and Fisher (1984).

gG glycoprotein G, Rixon and McGeoch (1985).

gD glycoprotein D, Watson et al. (1983)

gE glycoprotein E, Rixon and McGeoch (1985).

ori origin of replication, ori_S, Stow and McMonagle (1983), ori_L, Quinn and McGeoch (1985).

PRV has only one major IE gene which maps within the repeat region (figure 2) (Feldman et al., 1979). This gene shares homology with IE gene 3 of HSV-1 (Davison and Wilkie, 1983b; Ben-Porat et al., 1983b) and encodes a polypeptide of molecular weight 180,000, which is believed to be equivalent in function to Vmw175. Studies with a mutant, tsG, which possesses a defect in the PRV IE gene product (IEP) show that this polypeptide is required for the transition to early stages of gene expression and for the repression of IE gene transcription (Ihara et al., 1983). In addition, IEP is capable of activating transcription of other eukaryotic genes and has been used to study the phenomenon of trans-activation (Green et al., 1983; Imperiale et al., 1983; Everett, 1984b). A detailed transcription map of PRV comparable to that of HSV is not yet available . However, a number of PRV genes have been mapped by marker rescue of mutants (Ben-Porat et al., 1982, Ihara et al, 1983) or by comparison of sequence data with those of HSV-1 (Robbins et al., 1986a). PRV and HSV have a colinear arrangement of genes encoding similar functions with the exception of approximately 40kb which is inverted in PRV with respect to HSV (Ben-Porat et al., 1983b; Davison and Wilkie, 1983b) as shown in figure 2.

2.2.4 Sequences involved in the constitutive expression of HSV genes

Promoters of HSV genes from all transcriptional classes are able to direct transcription in the absence of viral infection. This applies to promoters of IE genes (Post et al., 1981; Mackem and Roizman, 1982a and b; Cordingley et al., 1983) and of early genes, TK, gB, gC and MDBP (Macnab and Timbury, 1976; Reed et al., 1976; Dreesman et al., 1980; McKnight and Gavis, 1980). The situation with regard to late promoters is more contentious. Frink et al (1981) found that an uninfected "Manley" cell free system did not initiate detectable mRNAs from two model late promoters. Other workers, however, have found late genes to be expressed in in vitro systems and in uninfected cells (Read and Summers, 1982; Mavromara-Nazos et al., 1986). These

results imply that many, if not all, HSV promoters are recognised to some extent by unmodified RNA polymerase II. For this reason, HSV genes, notably that of the TK gene, have been used for the study of constitutive expression of certain classes of genes in eukaryotic systems. The fact that cloned HSV genes can be transcribed in the absence of superinfection does not correlate with the absolute requirement of early and late genes for the trans-activator, Vmw175, to facilitate their expression. An explanation of this apparent anomoly may be that HSV genes are maintained in a repressed state during the lytic cycle (Godowski and Knipe, 1986).

The sequence requirements for the expression of the TK gene in Xenopus oocytes have been finely mapped by McKnight and co-workers. Initial studies revealed that DNA sequences required for TK expression extended no further than 110bp from the mRNA initiation site (McKnight et al., 1981). In subsequent studies a series of linker scanning mutations were assayed for their effect on transcription. It was found that three spatially distinct transcription signals, referred to as the proximal signal and the distal signals (dsI and dsII), occur in the 105 nucleotides flanking the TK gene (figure 5). The proximal signal harbours the TATA homology and is essential for accurate initiation, while the distal elements act to augment transcription (McKnight and Kingsbury, 1982). The function of dsI is dependent on the integrity of dsII (McKnight et al., 1984). The distal signals each contain a copy of the hexanucleotide CCGCCC in opposite orientations. In addition, the pentanucleotide CCAAT is contained, in the inverted orientation within dsII. Both the CCAAT and CCGCCC motifs occur in other eukaryotic promoters and are binding sites for the cellular transcription factors, CTF and Sp1, respectively (as discussed in section 3.2).

A comparison of the promoter sequences of numerous HSV genes revealed that most possessed identifiable TATA boxes at around -30bp and a number also exhibited homologies to the CCAAT box at -60bp from the initiation sites (Wagner, 1985). Functional analysis of gD and IE promoters has shown

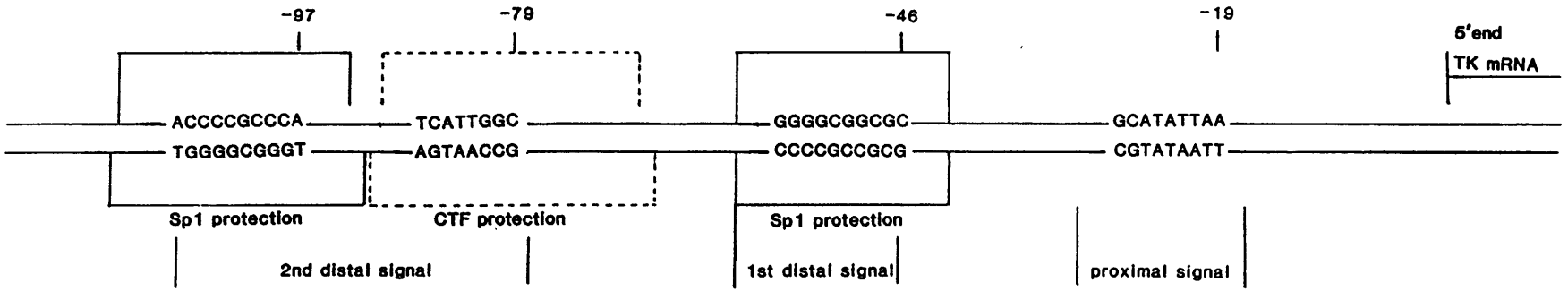
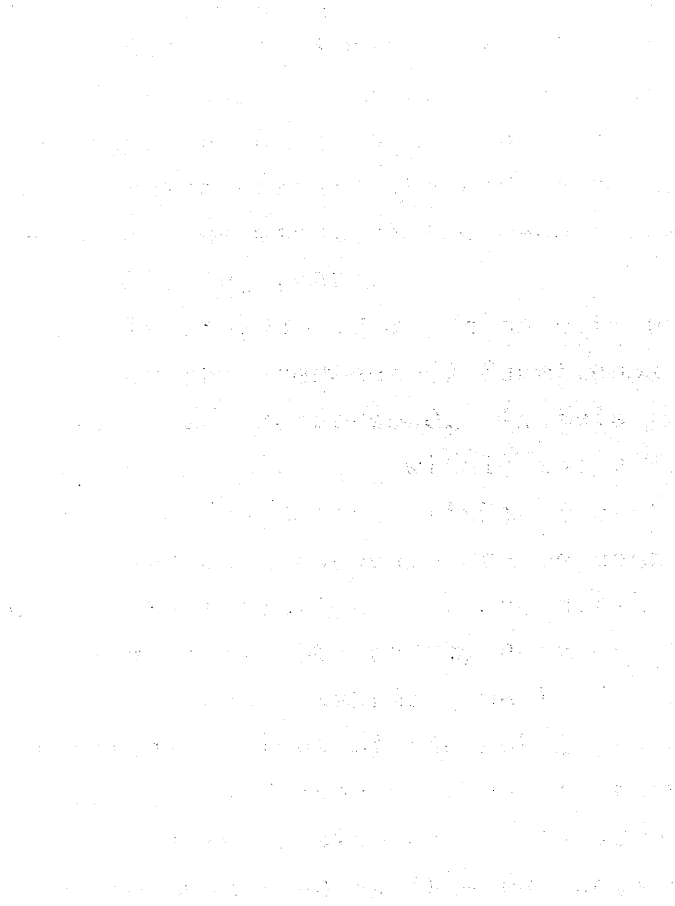

-97
-79
-46
-19
5'end
TK mRNA
ACCCCGCCCA
TCATTGGC
GGGGCGGCGC
GCATATTAA
TGGGGCGGGT
AGTAACCG
CCCCGCCGCG
CGTATAATT
Sp1 protection
CTF protection
Sp1 protection
2nd distal signal
1st distal signal
proximal signal

Figure 5. Promoter of the TK gene showing transcription factor binding sites.

The sequence and numbering is derived from McKnight et al. (1981). Brackets above and below the line designate approximate areas of DNAse I footprint protection (Jones et al., 1985). Important transcriptional control signals, as identified by sensitivity to base substitution mutagenesis are indicated (McKnight et al., 1984).



the importance of TATA and GC rich motifs for the transcription of these genes (Mackem and Roizman, 1982c; Cordingley et al., 1983; Everett, 1984a). It should be noted, however, that despite homologies to TATA, CCAAT and GC rich elements in the 5' flanking regions of HSV genes, there is a great deal of diversity even between promoters of the same transcriptional class (Wagner, 1985; Mackem and Roizman, 1982c). These differences could account for the variation in the level of expression of genes in equivalent groups, as observed by Honess and Roizman (1974), O'Hare and Hayward (1985a) and Harris-Hamilton and Bachenheimer (1985).

2.2.5 Cis and trans regulation of IE genes

There are five IE genes, the mapping and location of which are shown in figure 4. Immediate early genes 1 and 3 are located within the inverted repeats and are therefore present in two copies per genome (Watson et al., 1979; Anderson et al., 1980; Mackem and Roizman 1980; Rixon et al., 1982). IE genes 4 and 5 map partially within the short repeat and therefore share a common 5' terminus, promoter and upstream region. Both of these genes are spliced and the exons, which contain the coding sequences, are located in the unique segments of the genome (Watson et al., 1981; Rixon and Clements, 1982).

As previously stated, IE genes do not require de novo protein synthesis or the presence of functional Vmw175 in order to be efficiently transcribed. In this respect the regulation of IE genes is unique within the viral genome. The characteristics of regulation within the IE class would be expected to depend on sequences located upstream of the 5' termini of the transcription unit and direct evidence for this hypothesis was first obtained by Post et al., (1981). These workers constructed a hybrid gene by fusing the promoter and upstream regions of the HSV-1 IE gene 3 to the TK coding sequences. This construction was then inserted into TK^- virus at the usual site of the TK gene. The IE/TK hybrid was found to behave as an IE gene, showing that the sequences which mediate regulation lie upstream of IE gene 3. In addition, LTK^- cells were transformed to the TK^+

phenotype with the hybrid construct. Surprisingly, it was found that TK mRNA synthesis in these cells was stimulated by superinfection at NPT with an HSV-1 mutant possessing a ts defect in Vmw175, or with TK^- virus in the presence of cycloheximide. These results indicated that transcription of IE genes was strongly stimulated by a component of the virus particle. Subsequent studies by Batterson and Roizman (1983) showed superinfection with UV irradiated virus to be effective in regulating expression of the TK gene under IE control. A mutant, tsB7, is believed to have a block in uncoating of the capsid (Knipe et al., 1981; Batterson et al., 1983). Superinfection with this mutant at the NPT was able to elicit a stimulatory response, showing that the trans-acting component was not located in the nucleocapsid. A number of viruses were screened for their ability to stimulate expression from the IE promoter. HSV-2 gave a positive response, while PRV, HCMV and adenovirus 2 were ineffective (Batterson and Roizman, 1983).

The studies identifying the trans inducing factor, now termed TIF, as Vmw65, a major tegument phosphoprotein (Campbell et al., 1984) are presented in section 6 of this thesis.

Concurrently, research has been carried out in order to characterise further the cis-acting sequences involved in the regulation of the IE genes.

Regions upstream of all five IE genes mediate stimulation by the TIF in TK^+ transformed cell lines or in transient assays (Mackem and Roizman, 1982a, b and c; Preston et al., 1984). Quantitation of mRNA levels confirmed that activation occurred at the level of transcription (Lang et al., 1984; Preston et al., 1984).

Deletion analysis of the upstream sequences of IE gene 3 showed this region to consist of two distinct domains and that a promoter element located downstream of -110 was capable of mediating constitutive expression, but did not respond to stimulation by the TIF. Sequences between -110 and -330 responded to transactivation, but required a promoter for their function (Cordingley et al., 1983; Mackem and Roizman, 1982a; Lang et al., 1984). The far upstream

sequences of IE gene 3 were also found to possess enhancer-like properties and were able to stimulate gene expression in the absence of superinfection (Lang et al., 1984; Preston and Tannahill, 1984).

A comparison of IE gene regulatory regions revealed the presence of a consensus sequence present in one or more copies upstream of all IE genes. The sequence of the consensus is GYATGNTAATGARATTCYTTGNGGG, the core, TAATGARATTC, being the most highly conserved part (Mackem and Roizman, 1982c). A similar distribution of sequences showing homology to this element also occurred in the upstream of HSV-2 IE genes (Whitton et al., 1983; Whitton and Clements, 1984).

Functional analysis has shown that the TAATGARATTC element is essential in mediating a response to the TIF (Preston et al., 1984; Kristie and Roizman, 1984; Gaffney et al., 1985; Bzik and Preston, 1986). All workers, however, found that this AT rich element alone was less effective than longer upstream regions and proposed that flanking regions were also involved, GC rich hexanucleotides being the most obvious candidates. In order to elucidate the the situation Bzik and Preston (1986) performed a series of deletions from either end of the far upstream region of IE gene 3. The TAATGARATTC element was found to respond fully to the TIF when flanked by GA rich sequences, which, in addition, could activate a non-functional homologue of the TAATGARATTC. Three enhancer elements, found to be separable from the regulatory motif were also identified. Two of these elements corresponded to the hexanucleotide GGGCGG or its complement, which are Spl binding sites (Jones and Tjian, 1985) and the third element contains the sequence GCGGAAC which shares homology with SV40, adenovirus and polyoma virus enhancer motifs (Weiher et al., 1983; Hearing and Shenk, 1983a; Herbommel et al., 1984), which also bind a cellular factor (Davidson et al., 1986; Wildeman et al., 1986).

In summary, induction of IE gene transcription by the TIF, Vmw65, is mediated by the TAATGARATTC homology in association with GA rich flanking sequences, located in the

upstream region of the gene. Expression of IE gene 3 is also stimulated by elements with enhancer-like properties.

2.2.6 Sequence requirements for the trans-activation of early genes

Expression of the HSV-1 early genes, although detectable in uninfected cell culture assays is strongly enhanced by cotransfection with plasmids encoding IE polypeptides, Vmw175 or Vmw110 (Everett, 1984b; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986). In the lytically infected cell there is an absolute requirement for functional Vmw175, as mutants with a ts lesion in this polypeptide are unable to transcribe early and late genes at NPT (Preston, 1979a, Knipe *et al*., 1981).

The effect of deletion and substitution mutations on the *trans*-activation of the TK promoter in transient expression assays and transformed cell lines has been analysed (Zipser *et al*., 1981; Smiley *et al*., 1983; Eisenberg *et al*., 1985; El Kareh *et al*., 1985). Sequence requirements for induction in HSV infected cells were very similar to those previously identified as promoter constituents in *Xenopus* oocyte assays (McKnight *et al*., 1981; McKnight and Kingsbury, 1982). However, Eisenberg *et al* (1985) noted that mutations in the region -61 to -79 had a greater inhibitory effect in the infected cell system, but as deletions in this region did not prevent *trans*-activation the authors proposed that induction of expression of the TK gene was not mediated by any specific sequence element. Coen *et al* (1986) introduced a series of linker insertion mutations of the TK promoter into the viral genome, then assayed their effect on transcription rate and mRNA accumulation. No induction-specific domain of the TK promoter was identified.

Studies of another early promoter, that of the gD gene, revealed that the same sequences were required for *trans*-activation by viral superinfection as for *cis*-activation by the SV40 72bp enhancer element (Everett, 1984).

The experiments described above argue against a model in which viral polypeptides effect trans-activation by binding directly to a domain of early promoters which is not recognised by cellular transcription factors. Coen et al (1986) observed that mutations in the TATA box of the TK gene drastically reduced transcription in infected cells indicating that the interaction of a trans-activator with a TATA binding polypeptide may be involved in the stimulatory response. Further evidence for this hypothesis was provided by Everett (in press). Hybrid constructs containing the SV40 early gene promoter respond poorly to superinfection with HSV-1, but activity was restored by replacing the SV40 TATA element with that of the HSV-1 gD gene.

2.2.7 Sequence requirements of late genes

Expression of true late (γ_2) genes is severely reduced under conditions which prevent DNA replication (Jones and Roizman, 1979; Holland et al., 1980) but is detectable using sensitive assays (Godowski and Knipe, 1985; Johnson et al., 1986). Silver and Roizman (1985) reported that the TK gene under the control of the upstream region of a γ_2 gene was regulated as a late gene when recombined into virus. However, expression of this hybrid construct exhibited similar kinetics to that of an early gene when integrated into the cell genome.

The transcription pattern of the late gene, US11 in transient assays was found to be comparable to that occurring in the infected cell and to be similarly dependant on DNA replication (Johnson and Everett, 1986a). Sequences involved in late gene expression were therefore investigated in plasmids containing replication origins. US11 and gC genes required only sequences up to -33 from the initiation site in order to be efficiently expressed (Johnson and Everett, 1986b; Shapira et al., 1987). Furthermore, deletion of the promoter sequences to -33bp from the 5' end of the early gD gene resulted in the acquisition of late kinetics of expression. It was concluded the TATA homology was necessary for late gene expression as deletion of this sequence from the US11 promoter drastically reduces

transcription (Johnson and Everett, 1986b). The promoters of late genes, like those of early genes respond to trans-activation by IE gene products, Vmw175 and Vmw110, in transient expression systems (DeLuca et al., 1985; Mavromara-Nazos et al., 1986). Vmw63 may also be involved in trans-activation of certain late genes (Sacks et al., 1985; Everett, 1986; section 2.3.1).

In conclusion, efficient transcription of late genes requires viral DNA synthesis plus the presence of IE polypeptides. Cis-acting requirement appears to be minimal and sequences involved are situated close to the initiation site, with special requirement attached to the TATA box.

2.3 Herpesvirus polypeptides

2.3.1. IE polypeptides

The IE polypeptides of HSV-1, Vmw110, 63, 175, 68 and 12 are encoded by IE genes 1-5, respectively. With the exception of Vmw12 all of the HSV IE polypeptides are transported to the nucleus and are phosphorylated (Pereira et al., 1977; Preston, 1979b, Marsden et al., 1982).

Polypeptides Vmw175, 110, 63 and PRV IEP are involved in the regulation of gene expression. The regulatory role of Vmw175 was first elucidated by the use of ts mutants, as described previously (section 2.2.1). This role was subsequently confirmed by cotransfecting cells with plasmids carrying IE gene 3, along with genes under regulation of HSV promoters (Everett, 1984b; Gelman and Silverstein, 1985, 1986; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a and b). In addition, Beard et al (1986) found partially-purified Vmw175 increased transcription of the gD gene in an in vitro system.

Cotransfection assays also revealed that Vmw110, either alone or together with Vmw175, could increase expression of HSV genes (Everett, 1984; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985 a and b). However, a mutant virus, carrying a deletion in IE gene 1, although exhibiting a multiplicity dependance, has a normal polypeptide profile (Stow and Stow, 1986), indicating that

Vmw110 is not essential in tissue culture.

Sachs et al (1985) showed that mutants with ts defects in Vmw63 were deficient in the synthesis of late proteins and the regulation of IE polypeptide synthesis at NPT. The regulatory role of Vmw63 was confirmed by Everett (1986) who found Vmw63 to augment Vmw175 and Vmw110 in the the trans-activation of the major capsid protein, Vmw155. A viable mutant has been identified which lacks the gene encoding Vmw12 (Longnecker and Roizman, 1986), showing it to be a non-essential function.

Vmw175 may have a role in the replicative cycle besides trans-activation and autoregulation. DeLuca et al (1984) isolated ts mutants with defects in Vmw175, which are effective in induction but are unable to replicate DNA or to synthesise late polypeptides. Three forms of Vmw175 are apparent upon electrophoresis in SDS polyacrylamide gels. The fastest migrating form, a, is modified to forms b and c, which accumulate in the nucleus (Pereira et al., 1977; Fenwick and Roizman, 1977). Processing and transport of mutant forms of the polypeptide to the nucleus are impaired (Preston, 1979; Cabral et al., 1980). Functional Vmw175 also accepts poly(ADP ribosyl)ation in vitro (Preston and Notarianni, 1983).

In addition to the trans-activation of herpesvirus genes, Vmw175, Vmw110 and PRV IEP increase the synthesis of cellular genes. Cloned copies of PRV IEP positively regulate the synthesis of β globin and rat pre-proinsulin genes in transient expression systems (Gaynor and Berk, 1983; Green et al., 1983; Imperiale et al., 1983). In vitro transcription of a number of eukaryotic genes was higher in extracts of PRV infected cells than in mock infected extracts. The IEP was responsible for the stimulation, as the activity of extracts from cells infected with a mutant, tsG, which carried a mutation in the IE gene, was heat sensitive (Abmayr et al., 1985). Everett (1985) found that promoters of exogenous ξ and β globin genes were activated by cotransfection with HSV-1 IE genes 1 and 3, while transcription of the endogenous β globin was not affected. Genes transcribed by RNA polymerase III, Ad-2 VA1

and Drosophila tRNA, are also trans-activated by the PRV IEP (Gaynor et al., 1985).

The lack of specificity of activation by IE gene products, together with the failure to delineate an essential responding sequence, as discussed in section 2.2.6, imply that these trans-activators do not function by direct interaction with a DNA sequence element, but rather via a cellular intermediate. This conclusion is borne out by the observation that purified Vmw175 will only bind DNA in the presence of cellular components (Freeman and Powell, 1982). Recent studies have identified Vmw175 in DNA protein complexes occurring upstream of IE genes 1 and 3 (Kristie and Roizman, 1986a and b, Faber and Wilcox, 1986; Muller, 1987), and the early gene encoding gD (Beard et al., 1986). It has been proposed that the sequence 5' ATCGTCNNNNYCGRC 3' is required for the formation of Vmw175 complexes (Faber and Wilcox, 1986). However, the specificity of sequence elements involved in Vmw175 binding does not correlate with promoter mapping data, as the gD binding site (between -80 to -120) occurs in a region which is not involved in the transcriptional regulation of this gene (Everett, 1983, 1984a). One of the binding domains of IE gene 3 (-24 to +8) involves the same sequences as those required for the repression of IE gene 3 transcription (O'Hare and Hayward, 1987). The autoregulation of IE gene 3 may be mediated by binding of Vmw175 to the initiation site while trans-activation of gene expression occurs by a more indirect mechanism.

2.3.2 HSV and PRV enzymes and DNA binding proteins

A number of early genes specify polypeptides which have enzymic activity and are involved in nucleotide metabolism or the synthesis of viral DNA. The enzymes known to be virally encoded are thymidine kinase (Kit and Dubbs, 1963; Jamieson et al., 1976), alkaline exonuclease (Morrison and Keir, 1968; Moss et al., 1979), DNA polymerase (Keir et al., 1966; Chartrand et al., 1979, 1980), ribonucleotide reductase (Cohen, 1972; Dutia, 1983), dUTPase (Wohlrab and Francke, 1980; Preston and Fisher, 1984). In addition, a

putative protein kinase has been mapped by virtue of its sequence homology with other known protein kinase genes (McGeoch and Davison, 1986). Although these enzymes may duplicate cellular functions they frequently have different substrate specificities and optimum reaction conditions from their cellular counterparts.

Mutants are available in all of the enzymatic functions listed above, with the exception of the protein kinase, and have assisted in the characterisation of the enzyme and mapping of the encoding gene. Thymidine kinase and dUTPase are not required for virus growth in growing tissue culture cells (Dubbs and Kit, 1964; Fisher and Preston, 1986), while DNA polymerase, alkaline exonuclease and ribonucleotide reductase are essential as ts lethal mutants of these genes have been characterised (Moss et al., 1979; Chartrand et al., 1980; Dutia et al., 1983).

The major DNA binding protein is also required for DNA replication (Conley et al., 1981). It may be involved in the dissociation of double stranded DNA as Powell et al (1981) have shown it to reduce the melting temperature of poly(dA).poly(dT). Mutants with a lesion in this polypeptide are also found to be defective in the regulation of gene expression (Godowski and Knipe, 1983, 1985, 1986).

The locations of HSV-1 genes encoding functions involved in DNA replication are shown in figure 4.

A number of enzyme activities are found to be increased following PRV infection, including TK (Hamad et al., 1966), DNA polymerase (Halliburton and Andrew, 1976), ribonucleotide reductase (Lankinen et al., 1982), exonuclease (Keir, 1968) and protein kinase (Stevely et al., 1985). These enzymes have different affinities for substrates from both the host cell and HSV enzymes. The TK, DNA polymerase and DNA binding protein are virally encoded, as shown by the isolation of mutants in these functions (Ben-Porat et al., 1982, 1983a; Ihara et al., 1983). The map locations of encoding genes is shown on figure 2.

2.3.3 Structural polypeptides of HSV and PRV

Structural polypeptides of herpesviruses have been

identified by analysis of species present in preparations of purified virions. Difficulties with this approach include the possibility of viral non-structural and cellular contaminants. The occurence of polypeptides in different stages of processing also creates a problem in determining the number of viral species. HSV virions are reported to contain between 15 and 33 polypeptides (Spear and Roizman, 1972; Heine et al., 1974), while estimates for PRV vary between 20 and 40 species (Stevely, 1975; Ben-Porat and Kaplan, 1985).

Studies of herpesvirions have led to the identification of three groups of polypeptides: glycoproteins; capsid proteins and tegument proteins.

The glycoproteins were identified by their ability to incorporate radioactively labelled sugars. HSV-1 has a minimum of seven glycoproteins: gB, gC, gD, gE, gG, and gH and gI (Spear, 1976; Bauke and Spear, 1979; Frame et al., 1986). A further two open reading frames which have sequences characteristic of glycoproteins have also been identified within U_S (McGeoch, 1985). Glycoproteins gB, gC, gD, and gE are sulphated, the sulphation of gE being extensive (Hope and Marsden, 1983). The glycoproteins are integral membrane components and are probably the only polypeptides exposed on the surface of the virion (Roizman and Furlong, 1974; Olshevsky and Becker, 1970). It has been proposed that gB, gC and gD are involved in adsorption of the virions to the cell surface (Johnson et al., 1984). Glycoprotein gB is essential for fusion of the viral envelope and cell membrane (Little et al., 1981) and gE specifically binds Fc regions of IgG (Bauke and Spear, 1979). Mutants with a defect in gC frequently have a syncytial plaque morphology (Keller et al., 1970).

The polypeptide composition of capsids isolated from the cell nuclei has been determined. Empty capsids contain at least five polypeptides, while a further two species are only associated with capsids containing DNA (Gibson and Roizman, 1972; Heilman et al., 1979). One of these polypeptides, VP22a is processed to VP22, a species found on the surface of mature nucleocapsids (Gibson and Roizman,

1974; Preston et al., 1983).

The tegument is the layer located between the capsid and the envelope. Tegument polypeptides are released, following solubilisation of the virion with nonionic detergent (Lemaster and Roizman, 1980). Virion proteins not classified as capsid species or glycoproteins are frequently described as tegument constituents. Little is known concerning the organisation of polypeptides within this region. It can be inferred, however, from comparisons of the efficiency of extraction of various polypeptides, that some species are more tightly associated with the capsid than others (Roizman and Furlong, 1974; Spear, 1980).

Polypeptides of the pseudorabies virion may be similarly classified into glycoproteins, capsid and tegument components. The virion contains at least four major, sulphated glycoproteins (Kaplan and Ben-Porat, 1976). Three of the glycoproteins are found to be antigenically related to each other and occur in a cross-linked complex (Hampl et al., 1984). PRV nucleocapsids, lacking DNA are found to contain a minimum of three major and one minor polypeptides (Ladin et al., 1982) whereas eight species have been identified in preparations of full and empty capsids (Stevely, 1975). However, such preparations are likely to include differentially processed forms of the same polypeptides.

2.4 Replication of herpesvirus DNA and virion assembly

2.4.1 DNA Replication

A proportion of HSV or PRV DNA molecules circularise following their entry into the cell nucleus as shown by electron microscopy studies (Jean et al., 1977; Jacob and Roizman, 1977). Circularisation is thought to occur by direct ligation (Ben-Porat and Kaplan, 1985) and does not require a de novo viral function (Jean and Ben-Porat, 1976; Poffenberger and Roizman, 1985). After the onset of replication DNA molecules with loops and eyes are visible, while later in infection larger than unit length molecules are found in a head to tail configuration, indicating that

DNA replication occurs by the rolling circle mechanism (Jean et al., 1977; Jacob and Roizman, 1977; Ben-Porat and Veach, 1980).

2.4.2 Origins of DNA replication

It was deduced from electron microscopy studies and analysis of DNA contained within defective virus particles that HSV and PRV genomes contain three origins of replication, two within the diploid R_S sequences (ori_S) and one within U_L (ori_L) (Frenkel et al., 1976; Jean et al., 1977; Rixon and Ben-Porat, 1979; Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982) (Figure 4).

The cis-acting sequences essential for HSV DNA replication have been accurately located by assaying the ability of DNA fragments to induce replication of bacterial vectors when present in HSV infected cells. The ori_S sequences map to a 90bp fragment located between IE gene 3 and IE gene 4 (Stow and McMonagle, 1983). Localisation and sequencing of the ori_L proved difficult due to the inability to clone the DNA fragment containing this region. However, sequencing of the putative ori_L from the defective of strain Angelotti (Gray and Kaerner, 1984) and from uncloned HSV-1 DNA (Quinn and McGeoch, 1985) was achieved. Lockshon and Galloway (1986) succeeded in locating the HSV-2 ori_L by the method employed for HSV-1 ori_S and found it to be contained within a 241bp fragment located between the DNA polymerase gene and the MDBP gene. The outstanding feature of ori_S and ori_L sequences is their possession of near perfect AT rich palindromes of 25bp and 68bp respectively, which share strong sequence homology. It appears probable that the ability to form a cruciform structure is a functional requirement of the replication origin sequences.

2.4.3 Maturation of DNA

The maturation of replicated concatemeric DNA is associated with two events, namely cleavage to produce unit length, linear molecules and their subsequent encapsidation into virus particles. The ubiquitous presence of the HSV-1 'a' sequence in defective genomes indicated its involvement

in these events (Kaerner et al., 1981; Vlazny and Frenkel, 1981). Proof of the requirement of the 'a' sequence for encapsidation was obtained by Stow et al. (1983) by showing that its insertion into ori_S containing vectors would allow plasmid DNA to be packaged in HSV-1 infected cells.

The presence of the 'a' sequence at the termini of standard molecules indicates that it is the site at which concatemeric DNA is cleaved (Davison and Wilkie, 1981; Mocarski and Roizman, 1982). Varmuza and Smiley (1985) found that a single 'a' sequence inserted into the TK locus was processed to give rise to two new termini. They deduced that the termini of mature viral DNA molecules are produced by two distinct cleavage events and that the sequences between these sites are duplicated by the DNA maturation system. The HSV-1 'a' sequence is also found to be involved in the inversion of the genome to give four isomeric forms (section 1.3.2).

As previously stated PRV does not possess terminal repeats equivalent to the HSV-1 'a' sequence. However, fragments from both termini of the genome are required for the cleavage/encapsidation process and are found to be present in defectives (Wu et al.,1986).

2.4.4 Assembly of herpes virions

Cleavage of HSV-1 DNA is associated with the modification of the polypeptide P40 (VP22a) to forms of lower electrophoretic mobility (Preston et al., 1983). In PRV, a structural polypeptide of mw 35,000, thought to be equivalent to P40, is likewise required for DNA encapsidation (Ladin et al., 1982). Other HSV and PRV polypeptides are believed to be involved in the process as mutants from various complementation groups are defective in packaging DNA (Ladin et al., 1982; Addison et al.,1984)

Nucleocapsids containing full length DNA are enveloped at the inner lammella of the nuclear membrane (Roizman and Furlong, 1974; Vlazny et al., 1982), although de-envelopment and re-envelopment may occur at the outer lammella and endoplasmic reticulum, respectively (Stackpole, 1969).

3 REGULATION OF EUKARYOTIC TRANSCRIPTION

The data presented in this thesis concerns the transcriptional regulation of HSV and PRV genes. Since herpesvirus genes are transcribed by host cell RNA polymerase II and associated factors, studies relating to mechanisms controlling the initiation of eukaryotic genes are of relevance.

Regulation of initiation of transcription of eukaryotic genes is mediated by interaction of RNA polymerase and other factors with *cis*-acting sequences located in the non-coding regions. Traditionally these regions were classified as promoter elements necessary for constitutive expression, or regulatory elements required for modulation of expression. However, it now appears that in many instances division of upstream regions into separate promoter and regulatory regions is not possible.

The most highly conserved sequence element is the TATA box which appears to be essential for accurate initiation *in vivo* and *in vitro*. Two other elements which may be viewed as components of the constitutive promoter are the CCAAT and GC boxes. These motifs are of particular interest as the *trans*-acting factors which interact with them have been identified.

In addition to sequences required for constitutive expression, a number of genes possess regulatory elements which are frequently found to have enhancer-like properties and will stimulate expression from heterologous promoters by many orders of magnitude. Regulatory elements may be inducible in nature and increase or repress transcription in response to certain stimuli and many are active only in a specific cell type.

The aim of the following sections is to examine examples of the *cis*-acting sequences outlined in this summary and to discuss, where possible, data concerning the nature of interacting cellular factors and mechanisms by which regulation of transcription is attained.

3.1 Initiation of transcription and the TATA homology

RNA polymerase II generally initiates transcription at a single nucleotide or small cluster of nucleotides (Baker and Ziff, 1981). The first nucleotide is usually a purine which is flanked by pyrimidines and the consensus CAY often occurs (Corden et al., 1980).

Comparative studies of the promoter regions of eukaryotic genes revealed that the sequence TATA(A/T)A(A/T) or close homologue was very frequently found to occur approximately 25bp upstream from the site of initiation. This sequence element was therefore recognised as a putative control signal (Breathnach and Chambon, 1981).

A major role of the TATA homology appears to be the accurate positioning of the mRNA start site. In vivo studies performed on the promoters of the SV40 early and adenovirus E1A genes showed that deletion of intervening sequence between the TATA box and initiation sites resulted in transcription proceeding from novel start sites approximately 25bp downstream of the TATA homology. Furthermore, deletion of the TATA elements of these genes resulted in the creation of heterogeneous start sites over a region of around 30bp (Benoist and Chambon, 1981; Ghosh et al., 1981; Hearing and Shenk, 1983b).

In addition to being a requisite for accurate initiation, the TATA homology is necessary for the efficient transcription of many genes. In vitro assays showed the TATA consensus to be of overriding importance as mutations or deletions in this region abolish detectable transcription in the rabbit β globin promoter (Grosveld et al., 1981) and conalbumin promoter (Wasylyk et al., 1980). This result is probably due to the failure of the in vitro systems to recognise other cis-acting control elements. In vivo studies of the sea urchin histone H2A, adenovirus E1A and rabbit β globin promoters showed transcription to be reduced but not abolished by the deletion of the TATA box (Grosschedl and Birnstiel, 1982; Grosveld et al., 1982; Hearing and Shenk, 1983b).

The role of the TATA homology in initiation is believed to be mediated by the binding of transcription

factors. Davison et al. (1983) showed that fragments containing a functional TATA sequence could sequester one or more factors from partially purified Hela cell extract. These factors were required for the formation of stable pre-initiation complexes with DNA template. More direct evidence was obtained by Parker and Topol (1984a). These workers observed that a necessary initiation factor in Drosophila cell extracts interacted specifically with a region of Drosophila gene promoters which included the TATA homology. Binding, as assayed by DNAase I footprinting, occurred between -40 and +25 with respect to the initiation site. A purified transcription factor, TFIID was found to bind specifically to the TATA box region of the adenovirus major late promoter (Sawadogo and Roeder, 1985b). It appears probable that this interaction is the first step in the assembly of a stable initiation complex which requires a further two transcription factors, TFIIB and TFIIE, in addition to RNA polymerase II (Fire et al., 1982; Culotta et al., 1985; Sawadogo and Roeder, 1985a and b). It may be surmised that the conformation of the complex positions the RNA polymerase enzyme so that the initiation event occurs precisely, at a site 25bp downstream of the TATA homology.

3.2 CCAAT and GC rich elements

Analysis of control sequences of eukaryotic genes has identified two elements, CCAAT and GC rich boxes which mediate basal expression of the gene in the absence of induction. These elements are frequently located directly upstream of the TATA homology and are considered to be components of the constitutive promoter.

The CCAAT box (consensus RRCCAAT) was first identified as a conserved element in the upstream sequences of a number of eukaryotic gene families (Benoist et al., 1980; Corden et al., 1980; Efsratiadis et al., 1980). Studies by Dierks et al. (1983) and Grosveld et al. (1981 and 1982) revealed that the CCAAT box of the β globin gene was required for efficient transcription, as point mutations in this sequence significantly reduced mRNA synthesis in vivo.

GC boxes (consensus GGGCGG or the complement CCGCCC) frequently occur in multiple copies. They may be clustered as in the SV40 early promoter which contains six tandem GC boxes in the region 50-100bp from the initiation site (Fromm and Berg, 1982), or they may be spatially distinct, as in the HSV TK promoter (McKnight et al., 1981). The IE gene 3 of HSV-1 and the human metallothionein gene possess GC boxes in the far upstream regions, interspersed with other regulatory elements (Jones et al., 1985; Gidoni et al., 1984). Both the GC and the CCAAT boxes occur and are functional in both orientations (Kadonaga et al., 1986 Graves et al., 1986).

Evidence implicating GC boxes as transcriptional control signals was first obtained by Dynan and Tjian (1983a). These workers found that a factor (Spl) isolated from Hela cells induced transcription from the SV40 early promoter which contains six copies of the GC motif, but not from other promoters lacking these sequence elements. Subsequent experiments using the DNAase footprinting technique revealed that the Spl factor bound specifically to the GC repeats of the SV40 promoter (Dynan and Tjian, 1983b). Similar studies using the HSV TK gene showed that the binding of two different transcription factors occurred and that the Spl factors bound to each of the GC elements while a second factor (CTF) protected an inverted CCAAT motif located between the two Spl binding sites (Jones et al., 1985) (figure 5). The effect of a series of promoter mutations on the binding of these factors was also examined. The results showed a direct correlation between in vitro binding and the efficiency of transcription in vivo, as previously described by McKnight and Kingsbury (1982) McKnight et al. (1984), Eisenberg et al. (1985) and discussed in section 2.2.4, demonstrating conclusively that Spl and CTF are functional transcription factors. The Spl factor has been purified from human cells by sequence specific DNA affinity chromatography and was found to consist of two related proteins of 105 and 95 kilodaltons, both of which possessed DNA binding and transcription activating properties. A functional CCAAT binding factor

(CBP) has also been identified in rat liver extract (as opposed to Hela cell extract) (Graves et al.,1986), showing that recognition of the CCAAT element is not a species or cell type specific phenomenon. It is not yet clear whether CTF and CBP are the same polypeptide species as they have slightly different binding properties (McKnight and Tjian, 1986).

The Spl binding properties of a number of promoters have now been identified and a comparison of 19 binding sites revealed that the consensus extends beyond the GC hexanucleotide and can be represented as (G/T)GGGCGG(G/A)(G/A)(C/T) (Dynan and Tjian, 1985b; Kadonaga et al., 1986)

The mechanism by which Spl and CTF mediate their effect is not yet known. It appears probable, however, that the binding of these factors to promoter regions may influence the formation of stable initiation complexes.

3.3 Enhancer elements

3.3.1 Introduction

Enhancers are defined as cis-acting sequences which are able to potentiate a dramatic increase in transcription from homologous or heterologous promoters, independently of orientation, position and distance.

The first enhancer was identified as cis-acting sequences located upstream of the SV40 early promoter (Gruss et al., 1981). It was subsequently discovered that these sequences were able to stimulate transcription from a linked β globin gene by several orders of magnitude, at distances greater than 3kb, and when located downstream of the globin gene (Banerji et al., 1981; Moreau et al., 1981; Fromm and Berg, 1983).

Enhancer elements, with similar properties have now been identified in many viruses, including adenovirus (Hearing and Shenk, 1983a), polyoma virus (De Villiers and Shaffner, 1981), bovine papilloma virus (Lusky et al., 1983), the herpesviruses: HCMV (Boshart et al., 1985), MCMV (Dorsh-Hasler et al., 1985) and HSV-1 (Lang et al.,1984),

the retroviruses: Molony murine sarcoma virus (Levison et al., 1982), Rous sarcoma virus (Luciw et al., 1983), the human lymphotropic viruses I, II (Sodroski et al., 1984) and human immunodeficiency virus (Sodroski et al., 1985). Viral enhancers are frequently located upstream of an early gene (for example, HCMV IE or adenovirus EIA gene) which does not require the de novo synthesis of viral trans-activators in order to be efficiently transcribed. The possession of an enhancer element may provide these genes with a mechanism for successful competition for transcription factors.

A number of cellular genes are also regulated by enhancer or inducible enhancer elements, including the immunoglobin heavy and light chains (Banerji et al., 1983), the lysozyme gene (Theisen et al., 1986) and β interferon genes (Goodburn et al., 1985).

In the following sections a number of well characterised regulatory regions will be examined in order to illustrate some of the properties of enhancer and inducible elements.

3.3.2 Sequences involved in enhancer function

Sequences required to mediate cis-activation have been studied in the greatest detail in the enhancer of SV40. Enhancer activity is associated with a 72bp repeat sequence, which is located between the early and late transcription units. The repeat itself does not constitute enhancer function as only one copy is necessary for activity (Gruss et al., 1981) and sequences further upstream, with respect to the early gene initiation site are also involved (Benoist and Chambon, 1981; Weiher et al., 1983). The SV40 enhancer contains a number of sequence motifs, the integrity of which are required for full activity. The motifs are organised in three domains (Weiher et al., 1983; Herr and Clerk, 1986; Zenke et al., 1986) and enhancer activity, when destroyed by mutations in any of the domains may be restored by reduplication of the remaining motifs (Weber et al., 1983; Herr and Clerk,1986).

It is of interest to note that the SV40 motifs required for activity have been identified in several other

viral and cellular enhancers. For example, the repeated motif 5' GGTGTGGG 3' is a required element of the immunoglobin light chain enhancer (Picard and Schaffner, 1984) and bovine papilloma virus (Lusky et al., 1983) and the SV40 enhancer core element, TGG(A/T)(A/T)(A/T)G shares homology with sequences of polyoma virus (De Villiers and Shaffner, 1981), murine sarcoma virus (Laimins et al., 1982) and HCMV (Boshart et al., 1985) enhancers.

The HCMV enhancer, which is located upstream of the major IE gene, contains four families of repeated elements. In addition to the 18bp repeats which share homology with the "enhancer core" sequences HCMV also possesses conserved elements of 17, 19 and 21bp (Boshart et al., 1985). Enhancer activity is not destroyed by extensive deletions, nor is it attributable to any one sequence element (Stinski and Roehr, 1985). It is possible that the repeat sequences substitute for each other in order to maintain enhancer function.

The current view is that enhancers are modular in structure and are composed of a number of active elements, which may be interspersed with redundant information (Serfling et al., 1985). Essential enhancer motifs may serve as binding sites for trans-acting factors (as discussed in section 3.3.4) and homologous motifs belonging to enhancers of different viral species may interact with the same cellular factors.

3.3.3 Tissue specific enhancers

The fact that enhancers may be more or less active in different cell types was originally suggested by studies on polyoma virus. A number of host range mutants of this virus have been isolated which have stringent cell type specificities. Sequence analysis revealed that these mutants contained alterations within the enhancer region (Fujimura et al., 1981; Katinka et al., 1981; Tanaka et al., 1982). This finding indicated that polyoma enhancer activity was regulated by interactions with cell type-specific factors.

In general, viral enhancers are most active in cell

lines derived from their natural host, including those of SV40, polyoma, murine sarcoma virus, HSV and papilloma virus (De Villiers et al., 1982; Laimins et al., 1982; Lusky et al., 1983; Spandidos and Wilkie, 1983; Lang et al., 1984). These viruses have presumably evolved sequences which maximise their interaction with host cell factors.

Tissue specificity of enhancers has been clearly demonstrated in the cellular immunoglobin heavy (IgH) and light (IgK) chain enhancers, both of which are functional only in cells of B lineage (Banerji et al., 1983; Gillies et al., 1983; Queen and Baltimore, 1983). In the course of lymphoid cell differentiation functional immunoglobin genes are created by joining one of a large number of variable exons upstream of an intron and a constant exon region (reviewed by Tonegawa, 1983). Enhancer sequences have been identified within the intron and are involved in transcriptional activation of the promoter, which is located upstream of the variable region exon (Banerji et al., 1983; Queen and Balitimore, 1983). This was the first instance of an enhancer found to occur naturally downstream of the promoter. Recently, an enhancer element was identified 3' to the polyadenylation site of the β globin gene. This enhancer is also tissue specific and was found to be functional in erythrocytes but not in fibroblasts (Choi and Engel, 1986; Hesse et al., 1986).

3.3.4. Cellular trans-acting factors

Enhancers are believed to mediate transcriptional regulation via interaction with cellular trans-acting factors. This concept was first indicated by the cell specificity of enhancers, as discussed in the previous section. Further evidence for the interaction of cellular transcription factors with enhancer sequences was obtained by Scholer and Gruss (1984). These workers showed that fragments containing the SV40 enhancer sequence could successfully compete for cellular factors in vivo, but point mutants, defective in enhancer function were unable to compete. Direct evidence of binding to SV40 and polyoma enhancers was obtained by DNAase footprinting experiments

(Wildeman et al., 1986).

The most obvious explanation of the tissue specificity of enhancers is that necessary trans-acting factors are not present in non-permissive cells. However, the situation in practice appears to be more complex. Wild type polyoma enhancer is only active in differentiated, as opposed to undifferentiated embryonal carcinoma cells (Katinka et al., 1980). Factors from both cell types, however, specifically interact with the enhancer sequences as observed by DNAase protection studies (Fujimura, 1986).

In vivo and in vitro experiments have demonstrated the interaction of trans-acting factors with the IgH enhancer (Scholer and Gruss, 1984; Mercola et al., 1985; Church et al., 1985). At least three components are involved, one of which, NFA1, was observed to bind the sequence, ATGCAAA, of both immunoglobin and SV40 enhancers (Sen and Baltimore, 1986; Bohmann et al., 1987) but a different factor, NF2A was observed to bind the ATGCAAT motif only when B lymphocyte extract was used (Davidson et al., 1986; Landolfi et al., 1986; Staudt et al., 1986). A factor ubiquitously present in nuclear cell extracts was also observed to bind specifically to the immunoglobin enhancer element, GATGGCCGATC, but not to close homologues of this sequence (Sen and Baltimore 1986).

The IgK enhancer is not active in pre B lymphocytes. In vivo competition studies show necessary trans-acting factors are available in these cells but their activity appears to be prevented by labile inhibitory proteins (Wall et al., 1986; Kelly et al., 1985).

The results discussed indicate that an enhancer domain may interact with a variety of cellular factors, which may be general or type specific, the resultant activity being dependant on the particular interaction which has occurred (Voss et al., 1986).

3.3.5. Mechanisms of enhancer function

Enhancers elevate the rate of transcription by increasing the density of RNA polymerase II molecules transcribing a gene (Triesman and Maniatis, 1985; Weber and

Schaffner, 1985). A number of models have been suggested to explain the mechanism by which this change is mediated (Courey et al.,1986; Ptashne, 1986). One model proposes that enhancer protein factors interact directly with the initiation complex by looping out intervening DNA. Evidence for this theory comes from the finding that the activity of the SV40 enhancer is diminished by manipulations which place it on the opposite face of the DNA helix from the initiation site (Takahashi et al., 1985). However, Courey et al. (1986) showed that the integrity of the sequences located between enhancer and promoter was required for transcriptional stimulation to occur. This result argued against a "looping" model but supported the "scanning" hypothesis which proposes that the enhancer region serves as an entry site for one or more transcription factors which then bidirectionally track the DNA until an initiation site is located. Indirect evidence for this hypothesis is also provided by the observation that the SV40 enhancer will preferentially activate the closest of two promoters (Wasylyk et al., 1983; Kadesch and Berg, 1986). However, recent investigations revealed that tandem promoters were equally active in the presence of the IgH enhancer (Atchison and Perry, 1986). Obviously further investigations of a number of enhancers require to be carried out before a unified mechanism is proposed.

3.4 Inducible regulatory elements

3.4.1 Cis-acting sequences

A number of genes possess regulatory elements which will induce transcription in response to the appropriate stimuli. These elements may have enhancer-like properties and will confer inducibility on a heterologous promoter and may also exhibit a strong tissue specificity, as evident in the case of the β interferon upstream region.

Expression of heat shock genes in response to stress occurs in a wide variety of organisms and cell types (Corces et al., 1981; Pelham, 1982; Schlesinger et al.,1982) and is therefore not tissue or species specific. Deletion analysis

of the Drosophila heat shock protein (hsp70) promoter in Xenopus oocytes or monkey COS cells suggested that the heat shock response was mediated by an 11bp sequence located between -47 and -66 (Bienz and Pelham, 1982; Pelham, 1982; Mirault et al.,1982). This element appears to be conserved in all heat shock genes. The consensus sequence C-GAA-TTC-G is capable of conferring activity on a non-responsive promoter (Pelham and Bienz, 1982). However, studies by Dudler and Travers (1984) in which promoter mutants of the hsp70 gene were introduced into Drosophila suggest that additional upstream sequences (to -96), which contain an additional copy of the heat shock element are also required.

The human ß interferon gene is highly inducible by viral infection or by synthetic double stranded RNA (Lengyel, 1982). This activation is tissue specific and only genes located in fibroblasts are responsive (Cavalieri et al.,1977b; Havell et al, 1978). Induction is mediated, at least in part, by the sequence located between -37 and -77 from the initiation site (Zinn et al.,1983). This region has many typical enhancer properties and functions independently of distance and orientation (Goodburn et al.,1985). Further deletion analysis of the inducible region revealed that it is composed of a negative regulatory element (-45 to -55), which is responsible for repression of enhancement in uninduced cells, plus an element (-55 to -77) which mediates constitutive expression (Goodburn et al.,1986). Five sequences which share homology with the latter element have been identified in the far-upstream region (Goodburn et al.,1985). Although the upstream copies are not essential for a response in tissue culture, it is possible that they are required, like the upstream elements of the hsp70 gene, in their natural environment.

Other examples of regulatory upstream regions composed of a variety of sequence elements which fulfil different functions are found in the promoters of metallothionein (MT) genes. Following the discovery that MT genes were induced by heavy metals (Durnam and Palmiter, 1981), deletion analysis of upstream regions plus sequence comparisons of MT genes from different species identified

the consensus TGCRCYCG as the core of the responsive element. Five such elements were located upstream of the mouse MTI and MTII genes, the most proximal of which occurred at -40. Induction of a marker gene required at least two upstream copies of the consensus sequence (Searle et al., 1985). Karin et al (1984) identified a sequence element in the far upstream region of the human MT IIA gene as being responsive to glucocorticoids. This element does not overlap with the metal responding motifs or with Spl binding sites also found in this region (Gidoni et al., 1984).

The above examples illustrate the complexity of the upstream regions of cellular genes. Regulatory regions appear to be composed of a variety of cis-acting elements which may be positive acting or inhibitory in nature. Important elements may be duplicated throughout the region, interspersed with other sequences. In the examples cited there is no clear division between upstream sequences and the distal promoter and the regulatory elements in question may therefore be viewed as either inducible enhancers or integral promoter constituents.

3.4.2 Role of trans-acting factors in inducible gene regulation

Inducible regulatory elements, like the "classical" enhancers discussed in section 3.3, appear to mediate their effects via interactions with cellular factors. Such interactions may only occur under conditions of activation, as observed to be the case for the hsp70 promoter. This region is found to bind two different cellular factors, one of which protects the region containing the TATA box under all conditions, while the second, although ubiquitously present, only binds to the promoter under heat shock conditions (Parker and Topol, 1984b; Wu, 1984). Inducible sequences may also function by binding a repressor molecule which inhibits expression under non permissive conditions. The inhibitory element of the β interferon gene binds a factor in uninduced cells, which, upon induction by double stranded RNA, dissociates. This event is followed by the

binding of a different factor to a region further upstream, which is required for transcriptional activation (Goodburn et al., 1986; Zinn and Maniatis, 1986). These results show that both negative and positive regulation influence the transcription of the β interferon gene.

A well characterised example of a cellular trans-acting factor is that of the glucocorticoid receptor complex, which is formed by the association of the glucocorticoid hormone with a specific receptor protein (Yamamoto and Alberts, 1976; Anderson, 1983). Glucocorticoids were found to selectively stimulate transcription of stably integrated mouse mammary tumour virus (MMTV) DNA (Ringold et al., 1977; Ucker et al., 1981). This stimulation is mediated by the hormone receptor complex, which selectively binds in vivo and in vitro to glucocorticoid responsive elements within the MMTV DNA (Chandler et al., 1983; Payvar et al., 1983). It is possible that negative regulation also occurs in this system, as deletion of the regulatory region upstream of the LTR promoter results in the relief of repression in the absence of hormone. Sequence comparisons have revealed similarities between glucocorticoid and oestrogen receptor genes and the V-erb-A gene of erythroblastosis virus. The cellular counterpart, C-erb-A is now known to be homologous to thyroid hormone receptor and binding studies show it to be a receptor for thyroid hormone (Weinberger et al., 1986). The conserved regions are the proposed DNA and hormone binding domains of the proteins (Weinberger et al., 1985; Green and Chambon, 1986).

3.5 Viral trans-activators

A number of viruses specify proteins which possess the ability to stimulate gene expression. In most instances these trans-activating polypeptides are encoded by genes which are transcribed early in infection, in the absence of prior protein synthesis. The IE polypeptide of PRV and Vmw175, Vmw110 and Vmw63 of HSV-1 fall into this category of viral trans-activators and are discussed in section 2.3.1. Adenoviruses, papoviruses and human lymphotrophic viruses

also possess well studied examples of polypeptides which are able to regulate gene expression.

3.5.1 Adenovirus E1A polypeptides

The adenovirus transcription unit expresses two overlapping mRNAs which encode 234aa and 289aa polypeptides (Perricaudet et al., 1979) which are multifunctional. First, they positively regulate transcription from adenovirus promoters (Jones and Shenk, 1979b; Berk et al., 1979) and also from cellular promoters (Triesman et al., 1983; Green et al., 1983;). Secondly, they repress transcription mediated by enhancer elements (Borelli et al., 1984; Velcich and Ziff, 1985). They are involved in the immortalisation of primary cell lines (Haley et al., 1984; Montell et al., 1984), and also induce cellular DNA replication, probably by mediating gene expression (Kaczmarek et al., 1986).

Different domains of the E1A polypeptides are involved in trans-activation and repression or transformation functions (Moran et al., 1986; Lillie et al., 1986). The 234pp lacks the trans-activating domain and functions primarily as a repressor (Lillie et al., 1986). Both polypeptides are required to induce a fully transformed phenotype (Montell et al., 1984; Bos and Van der Eb, 1985)

Extensive promoter deletions have failed to reveal any specific sequence element required for E1A trans-activation other than those involved in promoter function in the absence of stimulation (Kingston et al., 1985; Murthy et al., 1985; Leff et al., 1985), indicating the lack of an E1A binding site. However, Kovesdi et al. (1986) showed that the promoter of the adenovirus E2 gene was protected from nuclease digestion by the binding of cellular factors and that binding was greatly increased in infected cells. It appears that the E1A product may elevate the transcription rate by increasing the affinity of cellular factors.

3.5.2 SV40 T antigen

The SV40 T antigen is required for efficient

transcription of late mRNA (Brady et al., 1984a; Keller and Alwin, 1984) and has an autoregulatory function (Khoury and May, 1977).

Purified T antigen binds specifically to three sites around the origin of replication (Tjian, 1978; Shalloway et al., 1980). Binding of the T antigen to the two sites closest to the early mRNA initiation site appears to repress the first phase of early gene transcription (Rio et al., 1980; Dimaio and Nathens, 1982). The increase in late gene expression does not appear to be totally dependent on T antigen binding, as genes under the control of late gene upstream sequences, but lacking the origin binding sites, are trans-activated by T antigen. A 33bp sequence located between the 72bp repeats is attributed with mediating this response (Keller and Alwin, 1985). T antigen also acts indirectly to induce cis-activaton by SV40 enhancer sequences of the non-responsive TK promoter (Robbins et al., 1986b). Trans-activation by SV40 T antigen, like that by adenovirus E1A and herpesvirus IE polypeptides appears to be indirect and to be mediated by interaction with cellular factors.

3.5.3 HIV tat and art

The retrovirus, human immunodeficiency virus (HIV) possesses two trans-activating polypeptides tat and art which are essential for the expression of structural genes and replication of the virus (Sodroski et al., 1985, Dayton et al., 1986; Fisher et al., 1986). It has been suggested that tat does not significantly affect mRNA levels but is required for the translation of the HIV polypeptides (Feinberg et al., 1986; Rosen et al., 1986). This proposed translational effect is consistent with the finding that sequences responsive to tat activation are located in the region of the LTR which contains the 5' mRNA leader (Rosen et al., 1985). However, increased levels of mRNAs were observed when heterologous genes under the control of the HIV LTR sequences were trans-activated by tat (Cullen, 1986). At present, the simplest explanation of tat action is that the binding of this protein to the mRNA causes both

a derepression of translation and an increase in mRNA stability.

The mode of action of the art trans-activator is also unclear. Sodroski et al. (1986) proposed that art relieves the post transcriptional block in the expression of the gag and env structural proteins, while Feinberg et al. (1986) found transcription of an art defective mutant resulted in an abnormal pattern of viral mRNA synthesis and proposed this gene to be "a trans-acting regulator of splicing".

MATERIALS AND METHODS

4 MATERIALS

4.1 Chemicals

Chemicals were purchased from BDH Chemicals UK, Pharmacia Fine Chemicals, Koch-Light Laboratories and Sigma (London) Ltd. Solvents were obtained from James Burroughs UK Ltd and Koch-Light Laboratories. Radiochemicals were purchased from Amersham International plc and synthetic oligonucleotides from New England Biolabs.

4.2 Enzymes

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

4.3 Cells

The cells used were BHK-21 clone 13 (Macpherson and Stoker, 1962) and PK15 supplied by Gibco Ltd.

4.4 Viruses

HSV-1 viruses used in this study were all derived from strain 17(syn$^+$) (Brown *et al.*, 1973) including the mutants tsK (Marsden *et al.*, 1976), TK$^-$ (Sanders, 1981), and tsK TK$^-$. Wild type PRV was used (Kaplan and Vatter, 1959).

4.5 Bacterial strains

Two bacterial strains of E.coli K12 were used: DH-1 (*rec*A1, *nal*A, r_R-, m_R-, *endo*I-, B-, *rel*A1)(Hanahan, 1983). JM101Δ (Lac', *pro*), *sup*E, *thi*$^-$, F'*tra* D36, *pro*AB, *lac*Iq, Z ΔM15 (Messing *et al.*, 1981).

4.6 Plasmids

HSV-1 genomic clones of the pGX series were obtained from stocks maintained at the Institute of Virology. EcoRI

fragments were cloned in pACYC184 or pBR328, KpnI fragments were tailed with dC and cloned into the gG tailed PstI site of pAT153. BamHI fragments were cloned in pAT153. PRV KpnIh was kindly provided by Dr. A. J.Davison and was constructed by the same procedure as HSV-1 cloned KpnI fragments. Plasmid pUC9 (Vieira and Messing, 1982) was utilised for most of the cloning operations carried out in the course of this work.

4.7 Monoclonal antibodies

Monoclonal antibodies against Vmw65, MA1044 and LP1 were kindly provided by Drs. J.W. Palfreyman and A.C. Minson, respectively.

4.8 Tissue culture media

Cells were grown in Glasgow modified Eagle's medium (Bus by et al., 1964) supplied by Gibco Ltd. Medium was supplemented with 100units/ml penicillin, 100ug/ml streptomycin.

ETC_{10}	Eagle's medium containing 10% tryptose phosphate, 10% calf serum.
EHU_2	Eagle's medium containing 2% human serum.
PBSA	170mM NaCl, 3.4mM KCl, 2mM KH_2PO_4 (pH7.2).
PBS	PBSA plus $CaCl2H_20$ and $MgCl_26H_20$ both at 1g/l.
Versene	0.6mM EDTA dissolved in PBSA containing 0.002% w/v phenol red.
Trypsin	0.25% w/v trypsin dissolved in Tris-saline.

4.9 Bacterial culture media

L.Broth	10g/l NaCl, 10g/l Bactopeptone. 5g/l yeast extract.
L.Broth agar	L.Broth plus 1.5% w/v agar.
2YT Broth	5g/l NaCl, 16g/l Bactopeptone, 10g/l yeast extract.

Top agar	8g/l NaCl, 10g/l Bactopeptone, 8g/l agar. Antibiotics were used in the following concentrations: Ampicillin, 50ug/ml; Tetracycline, 5ug/ml.

4.10 Commonly used buffers

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

Restriction enzyme buffers

low salt	10mM Tris HCl (pH7.4), 10mM $MgCl_2$, 1mM DTT.
medium salt	50mM NaCl, 10mM Tris HCl (pH7.4), 10mM $MgCl_2$, 1mM DTT.
high salt	100mM NaCl, 10mM Tris HCl (pH7.4), 10mM $MgCl_2$.
Sma	20mM KCl, 10mM Tris HCl (pH8.0), 10mM $MgCl_2$, 1mM DTT.

5 METHODS

5.1 Tissue culture

BHK and PK cells were grown in rotating plastic bottles (850cm^2) from a seed stock of approximately 6x10^7 cells at 37^{o}C in 100ml of ETC_{10} and an atmosphere of 5% CO_2 and 95% air. Two days later confluent monolayers were harvested in 10ml of trypsin/versene (1:1 v/v), 10ml of fresh ETC_{10} was added and cells were resuspended by pipetting. This suspension was used to seed subsequent monolayers.

5.2 Preparation of virus and viral proteins

5.2.1 Preparation of stocks of infectious virus

Wild type PRV, and mutant and wt HSV, were grown in BHK cells in glass roller bottles according to the following procedure: a 90% confluent monolayer was infected at moi of 0.003 pfu per cell in 40ml of ETC_{10} and incubated at 31^{o}C until the cells exhibited obvious CPE (2-3 days). The medium was removed and centrifuged at 3,000rpm for 15min. Cell associated virus (CAV) was prepared from the pellet by resuspending it in 2ml of ETC_{10} and sonicating to disrupt the cells. The supernatant from the 3,000rpm centrifugation step was used to prepare cell released virus (CRV). This medium was centrifuged at 15,000rpm for 2h at 4^{o}C in a GSA rotor, and the pelleted virus was suspended in 1ml ETC_{10} by sonication. Sterility checks were performed by streaking virus preparations on blood agar plates and incubating at 31^{o}C for 5 days. Virus preparations were titrated on BHK cells and were stored at -70^{o}C.

5.2.2 Particle counts

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

5.2.3 UV irradiation of virus

Approximately 10^9 pfu of virus was diluted in 10ml of Eagle's medium (without phenol red) and centrifuged at

15,000rpm for 1h in an SS34 rotor. The virus pellet was resuspended in fresh medium by sonication then maintained on ice until required. 0.8ml of the suspension was placed in a 50mm Petri dish and irradiated by a Englehard- Hanovia bacteriocidal UV lamp with an output of 32erg/mm^2/sec, at a distance of 14cm for periods of between 5 and 10min (Notarianni and Preston, 1982). Calf serum was then added to 10% and virus stored at -70^{o}C. The titre of the virus stock before and after UV irradiation was determined to ensure reduction of the order of 5x10^5 fold had occurred.

5.2.4 Preparation of ^{35}S labelled virions

A roller bottle of confluent BHK cells was infected with 2x10^8 pfu of wt HSV-1 virus in 20ml ETC10 and incubated at 37^{o}C for 6h. The medium was removed and replaced with 20ml ETC_{10}, containing 1/5th the normal methionine concentration, plus 2mCi ^{35}S methionine at 800Ci/mmol. The cells were incubated for a further 16h at 37^{o}C and then the medium was decanted and centrifuged at 11,000rpm for 5min in an SS34 rotor. The supernatant was layered gently over a sucrose cushion (5ml of 10% w/v sucrose, 10% v/v PBSA) and tubes spun at 18,000rpm for 2h at 4^{o}C. After washing, the pellet was resuspended in 0.5ml of 10% v/v PBSA. This preparation was used immediately or stored at -70^{o}C.

5.2.5 Extraction of virion tegument and envelope proteins

400ul of ^{35}S labelled virions was added to a solution containing 0.03% v/v NP40, 50mM NaCl, 10mM Tris HCl (pH7.5), 1mM EDTA in a final volume of 4ml. The virion suspension was incubated at 4^{o}C for 1h then spun at 30,000rpm for 1h at 4^{o}C in an AH650 rotor. The supernatant, which contained solubilised glycoproteins and tegument proteins was stored at -70^{o}C.

5.2.6 Calf thymus DNA cellulose columns

A 15% suspension (w/v) of cellulose powder (Sigmacell) was prepared in 1mg/ml double stranded calf thymus DNA. The suspension was spread over the inside of a

beaker, dried at 31°C and then lyophilised. 0.1ml DNA cellulose columns were prepared in 2ml syringes which were plugged with glass fibre paper. The columns were washed with 2ml MSB (50mM NaCl, 10mM Tris HCl (pH7.5), 1mM EDTA). All further procedures were carried out at 4°C. 1ml of virion extract (section 5.2.5) was added and the column washed with 1ml of MSB. Elutions were carried out with 0.5ml fractions of MSB containing increasing NaCl concentrations and the columns washed with a further 1ml of the same solution. 2ml of H_2O and 5mg of carrier protein were added to the eluates and flowthrough and the protein precipitated after the addition of 4 volumes of acetone at -20°C.

5.3 Construction, preparation and characterisation of plasmid DNAs

5.3.1 Restriction enzyme digestions

DNA was digested in a final volume of between 10 and 50ul in 0.01% BSA and low, medium or high salt or Sma buffer reaction conditions, as specified by Maniatis (1982) or as recommended by the manufacturers. The number of units of enzyme added was dependant on the activity of the enzyme and the amount of DNA present. Reaction mixtures were generally incubated at 37°C for 3h.

5.3.2 Separation of DNA fragments by non-denaturing gel electrophoresis

Agarose gels: 200ml horizontal slab gels (260mmx160mmx5mm) containing 1-1.5% (w/v) agarose were electrophoresed in E buffer plus 0.5ug/ml ethidium bromide, for approximately 16h at 40-80V.

Samples were loaded in 2% Ficoll, 20mM EDTA (pH7.5), E buffer and 20ug/ml bromophenol blue.

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

Polyacrylamide gels Vertical non-denaturing polyacrylamide gels were used for resolution of DNA fragments smaller than 0.5kbp.

A solution of 50ml containing 5-10% acrylamide (diluted from a stock of 29% acrylamide and 1% N,N-methylbisacrylamide), 0.55xTBE was prepared and 0.5ml 10% APS and 80ul TEMED added immediately prior to pouring into a prepared gel sandwich (260mmx160mmxlmm). Acrylamide was allowed to polymerise then samples were applied in 5% glycerol, 0.5xTBE, 20ug/ml bromophenol blue, 20ug/ml xylene cyanol. Electrophoresis was carried out in 0.55xTBE at 40-200V for 3-16h. Bands were visualised by soaking the gel in lug/ml ethidium bromide prior to UV illumination, or by autoradiography of radioactively labelled DNA.

5.3.3 Elution of DNA fragments from gels

A slice of agarose containing the required DNA fragment was removed from the gel. The DNA was isolated from the agarose by electroelution in 1xEEB at 30mA for 1-2h using wells designed for this purpose. The DNA was removed in 200ul of EEB and was purified by phenol/chloroform extraction and precipitation with an equal volume of isopropanol at room temperature in the presence of 4ug E.coli rRNA. A polyacrylamide gel slice containing the DNA fragment was cut into small pieces prior to incubation overnight in 600ul of acrylamide elution buffer (AEB) (0.5M sodium acetate, 0.1% w/v SDS) 2mM EDTA, 20mM Tris HCl (pH7.5) at 45°C. The sample was then centrifuged for 2min at 13,000rpm and the supernatant retained. The acrylamide pellet was incubated for a further 2h in 400ul AEB then centrifuged as before. Supernatants were pooled and filtered through Whatman GF/C paper in a 2ml syringe. The DNA was precipitated after the addition of E.coli rRNA in 2 volumes of ethanol at -20°C.

5.3.4 Ligation of DNA fragments to plasmid vectors

In most cases the vector used was pUC9 (Vieira and Messing, 1982) which contains a multilinker cloning site. Ligations were performed using 50ng of linearised vector and

200-500ng of purified fragment plus 1 unit of T4 ligase in 20mM Tris HCl (pH7.6), 10mM $MgCl_2$, 10mM DTT and 0.6mM ATP in a 4ul reaction at 15°C for 16h.

To prevent reannealing linearised vector was treated with bacterial alkaline phosphatase in 50mM Tris (pH8.5) at 65° for 1h or calf intestinal phosphatase in the same conditions as restriction enzyme digest. The sample was then phenol/chloroform extracted and precipitated prior to insertion of a DNA fragment.

Partial digestions of vectors were obtained by digesting 2ug samples of DNA in a series of ethidium bromide concentrations (3-30ug/ml) using various amounts of enzyme (0.3-10 units per reaction). Linear bands were eluted from 200ml agarose gels.

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

Phosphorylated restriction enzyme site linkers were inserted into vectors by the same procedure as DNA fragments. 0.15ug of linker was used per ligation.

5.3.5 Transformation of E.coli

10ml of L.Broth was inoculated with 5ul of glycerol stock of DH-1 and incubated at 37°C overnight. 1ml of this culture was added to 90ml of L.Broth and the mixture shaken at 37°C until the OD_{600} was 0.2 (normally for 2.5 h). After cooling on ice for 10min the culture was centrifuged at 3,000rpm for 15min at 4°C in an SS34 rotor. Bacterial pellets were resuspended in a total of 50ml of ice cold 100mM $CaCl_2$ and incubated on ice for 1h. This mixture was centrifuged as before and E.coli suspended in 1ml of ice cold $CaCl_2$. 100ul $CaCl_2$ shocked cells was added to 2ul of ligation mix (section 5.3.3) and incubated on ice for 1h. The mixture was then heated at 42°C for 2min and added to 2ml of L.Broth. These cultures were agitated at 37°C for 90min and 200ul aliquots were spread on L.Broth agar plates

containing the appropriate antibiotic. Plates were incubated at 37°C overnight.

5.3.6 Analysis of transformed E.coli colonies

Colonies were picked from agar plates into 2ml of L.broth plus antibiotic and shaken at 37°c for 6h. Choramphenicol was added to a final concentration of 25ug/ml and incubation continued overnight at 37°C. The following day the E.coli cells were pelleted at 5,000rpm for 5min in an MSE microfuge and the pellet was resuspended in 100ul STET (8% w/v sucrose, 5% v/v Triton X100, 50mM EDTA, 50mM Tris HCl pH8.0) containing 1mg/ml lysozyme, or in 100ul TELT (50mM Tris HCl pH7.8, 62.5mM EDTA, 0.4% v/v Triton X100, 2.5M LiCl) plus 1mg/ml lysozyme. Following incubation at 100°C for 1min the mixture was centrifuged at 13,000rpm for 10min and pellets discarded. TELT preps were placed on ice for 5min prior to centrifugation. The supernatant was precipitated with 100ul of isopropanal at -20°C for 30min. After centrifugation at 15,000rpm for 5min the pellets were washed in ethanol, dried and resuspended in 30ul H_2O.

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

5.3.7 Large scale plasmid DNA preparations

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

5ul of glycerol stock of bacteria containing the appropriate plasmid was added to 10ml of L.Broth plus antibiotic and incubated at 37°C overnight. 0.5ml of this stock was used to inoculate 200ml of L.Broth containing antibiotic and the culture was shaken at 37°C for 8h. Chloramphenicol was then added to a final concentration of 25ug/ml and incubation continued overnight. The culture was then centrifuged at 8,000rpm for 10min in a GSA rotor. After decanting the supernatant the pellet was suspended in 5ml of 25% w/v sucrose, 50mM Tris (pH8.0), and 2.5ml of

10mg/ml freshly prepared lysozyme added. The samples were mixed then maintained on ice for 30min. 2ml of 250mM EDTA (pH7.5) was added and after a further 5min 1.5ml of 5M NaCl and 1.5ml of 20% w/v SDS was mixed with extract. Incubation on ice was continued for 2h and the preparations were centrifuged at 20,000rpm for 1h at 4°C in an SS34 rotor. The supernatant containing the plasmid DNA was further purified by two extractions with phenol/chloroform, one with chloroform followed by precipitation with two volumes of ethanol at -20°C. DNA was pelleted by centrifugation at 2,500rpm for 15min and dissolved in 10ml of TNE (20mM Tris (pH7.5), 100mM NaCl 1mM EDTA) plus 10ug/ml RNAase. After incubation at 37°C for 4h approximately 2mg of proteinase K was added and samples maintained at 37°C overnight. The proteinase K was removed by two phenol/chloroform extractions plus a chloroform extraction. Sodium acetate (pH7.0) was added to a concentration of 0.3M and DNA was precipitated by 0.5 volumes of isopropanol at room temperature for 2h. After centrifugation at 5,000rpm for 15min at 20°C in an SS34 rotor the pellet was washed with ethanol, dried and dissolved in 500ul H_2O.

5.3.8 Estimation of DNA concentration

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

5.4 Gene expression experiments

5.4.1 Transfection of DNA into cells

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

Cocktails were prepared containing chimaeric TK plasmid, cloned viral genomic DNA (where applicable) plus carrier DNA to a total of 3ug, 66ul of 2xHeBS (260mM NaCl,

9.8mM KCl, 1.6mM Na_2HPO_4, 11mM D glucose, 42mM HEPES (pH7.05) and distilled water to 132ul. 9ul of 2M $CaCl_2$ was then added and samples immediately vortexed for 20s. The tubes were allowed to stand at room temperature for 30min. The medium was then removed from 80% confluent monolayers grown in 30mm Petri dishes and they were overlaid with the calcium phosphate precipitate and incubated at 37°C for 40min, with intermittent rocking.

Plating medium containing 17.75ml ETC_{10}, 20ml of "conditioned" ETC_{10}, 2ml of 2xHeBS and 0.25ml of 2M $CaCl_2$ was prepared and 2ml was added to each monolayer and incubation continued for a further 3h at 37°C. The plating medium was then removed from the cells and 25% v/v dimethyl sulphoxide in 1x HeBS was applied for 4min at room temperature. The dimethyl sulphoxide was removed and cells were washed twice with ETC_{10}, a further 2ml of ETC_{10} was added and monolayers were incubated at 38.5°C for 1h. The medium was removed and cells were then either infected with UV inactivated virus or mock infected. After absorption for 1h fresh ETC_{10} was added and incubation continued at 38.5°C for 16h.

5.4.2 Preparation of cell extract

The medium was removed from the cell monolayers (transfected as above) which were then washed with ice cold PBS. 1ml of fresh PBS was added and cells were scraped into this medium. The cell suspension was transferred to 1.5ml reaction tubes and centrifuged at 5,000rpm for 1min. The supernatant was carefully removed and cells were resuspended in 100ul ice cold TK lysis buffer (20mM Tris HCl(pH7.5), 2mM $MgCl_2$ 10mM NaCl, 6.5mM β mercaptoethanol, 0.5% v/v NP40) by vortexing and the tubes placed on ice for 5min. Samples were centrifuged at 13,000rpm for 2min then supernatant transferred to fresh tubes and maintained on ice or stored at -70°C.

5.4.3 TK assays

Samples of cell extract were assayed for TK activity in a reaction mix containing 100mM sodium phosphate (pH6.0),

10mM $MgCl_2$, 50uM dTTP, 5mM ATP and 100uCi/ml ^3H thymidine at 47 Ci/mmol in a 50ul reaction mix at 30°C. In most experiments a 5ul sample of cell extract and a 30min incubation period was used. The reaction was terminated by the addition of thymidine to 33uM and tubes were heated at 90°C for 4mins. They were cooled on ice for 5min and centrifuged at 13,000rpm for 2min. 50ul of the supernatant was spotted onto a DE81 filter paper disc. The discs were washed three times in 4mM ammonium formate (pH4.0), 10mM thymidine at 37°C and twice in absolute alcohol. Discs were then dried under a heat lamp and placed in vials containing 5ml of scintillation fluid. Incorporation of ^3H thymidine was determined by scintillation counting and used as a measure of TK activity.

5.5 Mapping of mRNA

5.5.1 Extraction of cytoplasmic RNA

A roller bottle of confluent BHK cells was inoculated with $6x10^9$ pfu of HSV-1 or PRV in 25ml ETC_{10} and incubated at 37°C for 6h. When IE cytoplasmic RNA was required cells were infected in the presence of 200ug/ml cycloheximide.

After viral infection had proceeded for 6h, medium was decanted and cells were washed with ice cold PBS. All the initial steps in RNA extraction procedure were carried out at 4°C or on ice to minimise nuclease degradation of the RNA. Cells were scraped into 25ml cold PBS and the suspension was centrifuged at 2,000rpm for 2min at 4°C. Cell pellets were then resuspended in 5ml lysis buffer (0.2M Tris HCl (pH8.5), 0.14M NaCl, 2mM $MgCl_2$, 10ug/ml cycloheximide, 0.5% NP40) and pipetted 4 times through a 10ml pipette while on ice and centrifuged at 2,500rpm for 3min at 4°C. The supernatant was transferred to a flask containing 15ml of 3mM Tris HCl (pH8.5), 2mM EDTA and 0.2% SDS plus 15ml phenol/chloroform at room temperature. The contents were mixed by swirling then left for 10min with intermittent mixing before centrifugation at 2,500rpm for 10min at 20°C. The supernatant was removed and subjected to two further phenol/chloroform extractions, followed by a

chloroform extraction. NaCl was added to 0.1M and RNA precipitated by the addition of 2 volumes of ethanol followed by standing at -20°C overnight. The RNA was pelleted by centrifugation at 2,500rpm for 10min at 4°C, and washed in ethanol, dried, then dissolved in 250ul of distilled water.

5.5.2 Hybrid arrest of in vitro translation

5.5.2.1 Hybridisation of DNA fragments to RNA

5ug samples of plasmid containing an appropriate DNA fragment were digested with a suitable restriction enzyme, phenol chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 80ul of deionised formamide plus 6ul of 1M PIPES (pH7.4) and heated for 5min at 90°C. While samples were at this temperature 6ul (approximately 2ug) of cytoplasmic RNA and 8ul of 5M NaCl were added, rapidly mixed, and samples were immediately transferred to a water bath at 58°C and incubated for 1h. Control samples with unrelated DNA or lacking DNA were treated in a similar manner. Following incubation, NaCl was added to a concentration of 0.2M in a final volume of 400ul and samples were precipitated with 1ml of ethanol at -20°C then dissolved in 5ul of water. DNA/RNA hybrids were denatured by boiling for 1min.

5.5.2.2 In vitro translation

In vitro translation was carried out as described by Preston (1977) and using components provided by Dr. C.M. Preston. A cocktail was mixed by the ordered addition of the following components: 8ul of 2.5M HEPES (pH7.7.), 8ul of 50mM $CaCl_2$, 6ul of 10mM magnesium acetate, 4ul of 100mM amino acids mixture (- methionine), 2ul of 100mM spermidine, 2ul of 100mM DTT, 2ul of 60mM CTP, 24ul of BHK cell ribosomal salt wash fraction, 140ul of rabbit reticulocyte S100 fraction, 40ul of rabbit reticulocyte pH5.0 fraction, 20ul of 20x energy mix (20mM ATP, 2mM GTP, 100mg/ml creatine phosphate and 400ug/ml creatine phosphate kinase (pH7.5)and 4ul of 10,000 units/ml micrococcal nuclease. This mixture

was incubated at 21°C for 10min to allow nuclease digestion of the rabbit mRNA, and 10ul of 100mM EGTA and 50ul of ^{35}S methionine (0.6mCi) was added. 12ul samples of this reaction mix were added to 3ul samples of mRNA, DNA/RNA hybrid or denatured DNA/RNA hybrid. The reactions were incubated at 30°C for 2h and terminated by the addition of 15ul of 100mM EDTA, 200ug/ml RNAase, 1% w/v methionine. The polypeptides translated *in vitro* were analysed by SDS PAGE or by immunoprecipitation with monoclonal antibody.

5.5.2.3. Immunoprecipitation

In vitro translation mix or extract to be immune precipitated was diluted in extraction buffer to give a final concentration of 0.1M Tris HCl (pH8.0), 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate, 0.2mM phenylmethylsulphonylfluoride and incubated for 1h on ice. 1/25 volume of preimmune rabbit serum plus 1/5 volume of fixed *Staphylococcus aureus* was added to the labelled antigen, followed by incubation at 4°C for 30min. One half of the sample was added to 5ul of monoclonal antibody, while the remainder was added to 5ul of ascites fluid as a control. Samples were placed at 4°C for 3h and after the addition of 100ul of Staphylococcus incubation was continued for a further hour. The samples were then washed by suspending them in 600ul of cold washing buffer (0.5M LiCl, 0.1M Tris (pH8.0), 1% β mercaptoethanol) then centrifugation for 1min at 5,000rpm. The supernatant was discarded and the washing procedure repeated twice. Samples were resuspended in PAGE buffer, boiled for 5min and centrifuged for 5min, prior to electrophoresis on an SDS polyacrylamide gel.

5.5.2.4 SDS polyacrylamide gel electrophoresis

40ml of gel mix was prepared containing the required amount of 30% acrylamide/3% DATD to give a final concentration of between 8% and 10% acrylamide plus 1xGB (1.5M Tris HCl (pH8.9), 0.4% SDS). The acrylamide was activated by the addition 0.5ml 10% w/v of APS and 40ul TEMED and poured into a vertical gel mould (170mmx130mmx1.5mm). While still liquid the acrylamide was

overlaid with 1ml of 1xGB and allowed to polymerise for 1h.

24ml stacking gel solution was prepared, which contained 5% acrylamide, 0.5% DATD, 1xSGB (0.5M Tris HCl (pH6.7), 0.4% SDS, 0.5ml of 10% APS and 35ul of TEMED.

The buffer was removed from the surface of the running gel and after washing, the mould was filled with the stacking gel solution and a suitable teflon comb was inserted.

Samples were prepared by boiling for 5min in a denaturing buffer of final concentration 50mM Tris HCl (pH6.7), 2% SDS, 10% glycerol. Gels were electrophoresed for 3-4h in a buffer containing 50mM Tris, 65mM glycine and 0-1% SDS and fixed by soaking overnight in 25% methanol, 6% acetic acid. Gels containing low levels of radioactivity were shaken for 1h in EN^{3}HANCE, followed by 1h in H_2O prior to being dried onto Whatman 3MM paper under vacuum. Autoradiographs of gels were made using Xomat S or Agfa film.

5.5.3 S1 nuclease mapping mRNA 5' terminus

5.5.3.1 5' ^{32}P labelling of probe

10ug of plasmid DNA containing a suitable insert was digested with an appropriate restriction enzyme. Tris HCl (pH8.0) was added to a final concentration of 10mM together with 300 units of bacterial alkaline phosphatase and the sample was incubated at 65^{o}C for 1h. Following phenol/chloroform extraction and ethanol precipitation the DNA pellet was dried then dissolved in kinase buffer (70mM Tris HCl (pH7.6), 10mM $MgCl_2$, 5mM DTT). 50uCi of γ^{32}P dATP plus 2 units of T4 polynucleotide kinase were added and the mixture incubated at 37^{o}C for 3h. The labelled sample was loaded on a 5% non-denaturing acrylamide gel, as described in section 5.3.2. Bands were detected by autoradiography using Xomat-S film. The appropriate band was cut out and DNA extracted according to the method in section 5.3.3.

5.5.3.2 DNA/RNA hybridisation and S1 nuclease digestion

Approximately 0.5ug of 5' end labelled DNA was precipitated with 10ug of cytoplasmic RNA. The resultant pellet was resuspended in 20ul of 90% v/v deionised

formamide 0.4M NaCl, 40mM PIPES (pH6.8), 1mM EDTA and heated at 90°C for 5min. The samples were transferred rapidly to a water bath at 58°C and incubated for 16h. They were then placed on ice. 4,000 units of S1 nuclease was added in S1 buffer to give a final concentration of 25mM NaCl, 3mM sodium acetate (pH4.5), 0.1mM $ZnSO_4$ in a volume of 200ul.

The samples were incubated at 37°C for 90min. EDTA (pH7.5) was then added to a concentration of 20mM and sodium acetate (pH7.0) to 0.3M. The samples were phenol/chloroform extracted, ethanol precipitated and electrophoresed on a denaturing polyacrylamide gel along side ^{32}P labelled size markers.

5.5.3.3 Denaturing polyacrylamide gels

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

5.6 Southern blotting

5.6.1 Transfer of DNA fragments to nitrocellulose

The method used was essentially that of Southern (1975). The agarose gel to be blotted was shaken for 45min in Gel Soak I (200mM NaOH, 600mM NaCl) then for a further 45min in Gel Soak II (1M Tris HCl (pH8.0), 0.6M NaCl) at room temperature. The gel was then transferred to two sheets of Whatman 3MM filter paper, supported by a glass plate. The edges of the filter paper were dipping into a tray containing 6xSSC. A sheet of nitrocellulose, the same dimensions as the gel was placed on top, covered by 2 sheets of 3MM paper and a stack of paper towels, also cut to size.

The towels were weighted and the blot left overnight in order for the DNA to transfer to the nitrocellulose. The following day the nitrocellulose was removed and allowed to dry in air and baked at 72°C for 2h.

5.6.2 Preparation of ^{32}P labelled probe

DNA fragments were labelled by ^{32}P as described by Rigby et al. (1977). 0.2ug of plasmid DNA was incubated in a reaction mix containing 40mM ATP, 40mM dTTP, 2.5ul of 10xNTB (5M Tris HCl (pH7.5), 1M $MgCl_2$, 1M DTT and 5mg/ml BSA) and $2x10^{-4}$ug DNAase at room temperature for 3min. The mix was then placed on ice and diluted to 25ul by the addition of 30uCi of both $\alpha^{32}P$ dGTP and $\alpha^{32}P$ dCTP, 3 units of E.coli DNA polymerase I plus distilled water. The reaction was incubated at 15°C for 2h. ^{32}P labelled DNA was then separated from unincorporated triphosphates by fractionation on a 10ml Sephadex G50 column. Elution of the column was carried out by addition of 10mM Tris/1mM EDTA and the first peak of radioactivity, which contained the DNA was collected. The specific activity was found to be approximately 10^8cpm/ug DNA.

5.6.3 Hybridisation to nitrocellulose filter.

DNA hybridisations were carried out under conditions of high stringency by the method of Southern (1975). The nitrocellulose containing separated DNA fragments was prehybridised in 100ml 6xSSC, 5xDenhardts (0.5% w/v Ficoll, 0.5% w/v polyvinylpyrollidone, 0.1% w/v SDS 0.5% w/v BSA) and 20ug/ml denatured calf thymus DNA at 70°C for 2h in a sealed polythene bag. Meanwhile the probe was denatured by incubation in 0.2M NaOH for 10min at 20°C, followed by neutralisation by the addition of 0.2M HCl.

The pre-hybridisation mixture was removed and replaced with the denatured probe in a mix of same final composition as the pre-hyridisation solution. The nitrocellulose filter was shaken overnight at 70°C then washed in 40xDenhardts, 4xSSC for 30min at room temperature, followed by three washes in 0.3xSSC, 0.1% SDS at 65°C. It was then air dried and autoradiographed.

5.7 DNA sequencing

Sequencing was carried out by the dideoxy/chain termination method (Sanger et al., 1977, 1980) using single stranded template obtained by the cloning of DNA fragments in M13 vectors (Messing et al., 1977; Messing and Vieira, 1982).

5.7.1 Cloning of DNA fragments into M13 vectors

A mixture of restriction fragments or single fragment isolated from a gel were ligated into the multilinker site of M13 mp8, mp18 or mp19 RF vectors (Messing and Viera, 1982) using the same procedure as described in section 5.3.4. Ligation mixes were transfected into E.coli strain JM101. Induction of competence of the JM101 bacteria was by the method described for DH-1 in section 5.3.5 except that JM101 were grown in 2YT Broth.

2ul of the ligation reaction was mixed with 100ul of competent JM101 and incubated on ice for 45min. The mixture was then heated at 42°C for 3min before adding it to 3ml molten top agar containing 208ug/ml isopropyl-D-thiogalactosidase and 208ug/ml 5-bromo, 4-chloro, 3-indolyl, B-D-galactopyranosidase plus 100ul of growing JM101 culture. The agar mixture was shaken and quickly poured onto L.Broth agar plates. After cooling the plates were incubated at 37°C overnight.

Plaques resulting from intact M13 phage vectors were blue in colour, while those produced by M13 vectors containing an insert were colourless allowing them to be easily identified.

5.7.2 Preparation of single stranded template

Colourless plaques were picked into 2ml aliquots of 2YT Broth containing 20ul of JM101 culture, and shaken at 37°C for 6h. The contents were poured into 1.5ml reaction tubes and spun at 13,000rpm for 5min. Supernatants containing the phage, were decanted into tubes containing 100ul of 20% PEG 6,000, 2.5M NaCl. The contents were mixed thoroughly and tubes incubated at 4°C overnight. Phage was

pelleted by centrifugation at 13,000rpm for 5min and the supernatant was completely removed. The pellet was resuspended in 100ul TE (10mM Tris HCl (pH8.0), 1mM EDTA) and extracted with 100ul phenol/chloroform. Following centrifugation at 13,000rpm for 5min the aqueous phase was removed, made to 0.3M sodium acetate and ethanol precipitated. DNA was washed in 80% ethanol, dried, and dissolved in 50ul TE.

5.7.3 Sequencing reactions

Sequencing reactions were based on the protocol of Sanger et al. (1977).

The primer was hybridised to the DNA templates as follows: 2ul of template plus 1ml of primer (0.2 pmol/ul) were mixed in a 10ul reaction containing 100mM Tris (pH8.0), 100mM $MgCl_2$ and incubated at 37°C for 30min. 2ul of this annealing reaction was added to each of four tubes containing 2ul of either A,T,G or C mix followed by addition of 2ul of Klenow mix. The composition of A,T,G and C mixes is shown in table 2. Klenow mix was freshly prepared and contained 2ul of $\alpha^{32}P$ dATP, 20 units of Klenow polymerase and was made to the required volume with 5.9mM dATP/5mM Tris HCl (pH8.0), 0.5mM EDTA. After a brief centrifugation to pool components in the bottom of the tube, the samples were incubated at 31°C for 15min. 2ul of "chase" mix (all dNTPs at 0.25mM) was then added and incubation continued for a further 30min. Samples were then applied to a denaturing gel.

5.7.4 Denaturing sequencing gels

Sequencing gels required prepared "top" gel and "bottom" gel mixes.

Top gel mix: 6% acrylamide, 0.3% N,N'-methylbisacrylamide, 9M urea, 0.5XTBE.

Bottom gel mix: 6% acrylamide, 0.3% N,N'-methylbisacrylamide, 9M urea, 2.5XTBE, 5%sucrose.

Gradient sequencing gels were poured as follows: 80ml of top gel mix was activated by the addition of 90ul of 25% APS plus 90ul of TEMED and 50ml of bottom gel mix was

activated by the addition of 13ul of 25% APS and 13ul TEMED 15ml of the polymerising top gel mix, then 12ml of the bottom mix was drawn into a 60ml syringe and added to a gel mould (400mmx430mmx0.35mm). The mould was then filled with the remainder of the activated top gel mix.

2ul of formyl dye (1mg/ml bromophenol blue, 1mg/ml xylene cyanol, 10mM EDTA in deionised formamide) was added to each sequencing reaction and they were heated at 90°C for 5min prior to loading on a gel. The gel was electrophoresed for 2h at 70W in a kit containing 1xTBE in the bottom well and 0.5xTBE in the top well.

Alternatively, samples were electrophoresed on a 6% single concentration acrylamide gel prepared from top mix alone. These gels were electrophoresed for 20min prior to loading of samples. The tank buffer was 0.5xTBE.

5.7.5 Handling of sequence data

The sequence data was managed by programs of the Staden database package (Staden, 1979, 1980). All computer analysis was carried out using a Digital Research PDP 11/44 machine, running under the RSX-11M operating system. Programs were implemented by Dr. P. Taylor.

Gel readings were entered into files using the BATIN program. They were then inserted in the database automatically using the DBAUTO program, or screened to determine overlap with existing database consensus sequence by DBX. The relationships between files was maintained by the DBU program which also incorporates facilities for editing database and entering readings manually.

Sequence homology was determined by the matrix comparison program "CINTHOM" (Pustell and Kafatos, 1982).

Table 2. Nucleotide sequencing mixes

Deoxynucleotide Triphosphate Mixes

	dA-0	dT-0	dC-0	dG-0
5mM dTTP	20	1	20	20
5mM dCTP	20	20	1	20
5mM dGTP	20	20	20	1
10xTE	50	50	50	50
H_2O	540	370	370	370

Sequencing Mixes

	dN-0	ddNTP	H_2O
T mix	200	200ul 0.3mM ddTTP	0
C mix	200	56ul 70uM ddCTP	144
G mix	200	100ul 0.1mM ddGTP	100
A mix	200	100ul 70uM ddATP	100

Numbers are in ul.

RESULTS AND DISCUSSION

6 MAPPING OF THE GENE WHICH ENCODES THE HSV-1 TRANS-ACTIVATOR

6.1 Strategy

As discussed in section 2.2.5, transcription of HSV-1 IE genes is stimulated by a component of the virion. The primary objective of this project was to identify and characterise the polypeptides involved.

Previous studies had shown that a DNA fragment which encoded the HSV-1 *trans*-activator Vmw175, could stimulate expression of the plasmid pTK1 which contained the coding and regulatory sequences of the TK gene, when both plasmids were cotransfected into BHK cells (Campbell and Preston, unpublished results). A similar approach was therefore employed to identify the *trans*-inducing factor (TIF) responsible for the regulation of IE genes. Cloned HSV-1 genomic fragments were screened in a transfection system for their ability to stimulate expression of the TK gene under the control of IE upstream sequences.

6.2 Cotransfection of HSV-1 DNA fragments with an IE-TK hybrid plasmid

In the first experiment a series of cloned overlapping fragments, which spanned the HSV-1 genome, were transfected into BHK cells along with pTKN1, by the calcium phosphate precipitation method. The plasmid pTKN1 (figure 8) was constructed by M.G. Cordingley and contains the HSV-1 TK gene linked at +48 to IE gene 3 upstream sequences at +27 and extending to -4,500. The TK gene in this plasmid is regulated in an IE specific manner (Post *et al.*, 1981; Mackem and Roizman, 1982a; Cordingley *et al.*, 1983). The HSV-1 genomic fragments used are depicted in figure 6.

The aim of this experiment was to determine whether any of the HSV genomic fragments would express sufficient amounts of the HSV-1 TIF to mediate stimulation of TK activity.

The results of initial investigations are shown in

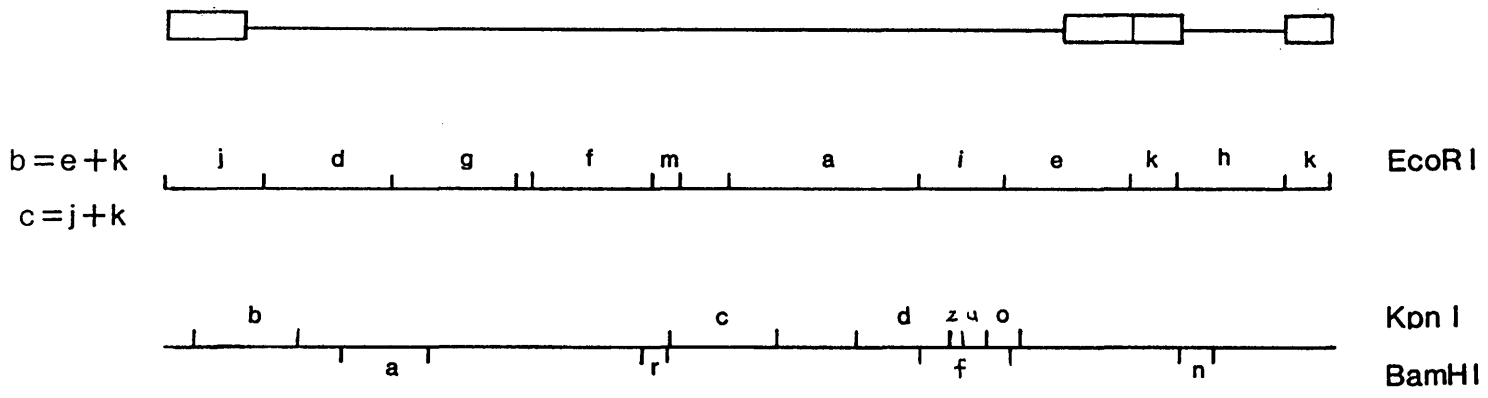

b=e+k
c=j+k
j
d
g
f
m
a
i
e
k
h
k
EcoR I
b
a
c
r
d
z
u
o
f
n
Kpn I
BamHI

Figure 6. Genome locations of cloned HSV-1 BamHI, EcoRI and KpnI fragments.

A diagram of the HSV-1 genome is shown above the restriction maps.

table 3. It can be observed that two of the HSV-1 fragments, EcoR I b and EcoR I i, induced elevated TK expression. Preliminary experiments revealed that the activity of the EcoR I i fragment was specific to IE regulated genes. Further experiments therefore focussed on delineating the coding sequences, located within this fragment, which were involved in producing the stimulatory response. Specificity studies of EcoR I b and derivatives of EcoR I i are described in section 6.6.

6.3 Location of the stimulatory sequences within the EcoR I i fragment

The trans-activating ability of EcoR I i was substantiated by the finding that two independantly isolated clones of this fragment, pGX63 and pGX69, induced a stimulatory response (table 4).

The locations of mRNAs encoded within EcoR I i, together with a restriction map of the region were determined by Hall et al. (1982) (figure 7). These data were later confirmed by nucleotide sequence analysis (Dalrymple et al., 1985; Pellett et al., 1985; Dalrymple, 1986). Using the mapping studies of Hall and co-workers as a basis, sub-clones of pGX63 were tested for stimulatory ability in cotransfection assays. Plasmids pGX158 and pMC2 contain the BamHI f and BamHI/EcoR I sub-fragments of EcoR I i, respectively. KpnI clones containing d, z and u fragments were provided by Dr. A. J. Davison.

It was observed that transfection with pGX158 gave rise to increased TK expression while KpnI d, z and u and pMC2 were ineffective (table 5 (table 3 for KpnId)). These results therefore localised the activity to an 8kb Bam HI fragment and suggested that one of the KpnI sites interrupted a DNA region necessary for stimulation.

6.4 Linker insertion mutants of BamHI f

Further delineation of sequences necessary for trans-activation was performed by constructing a series of plasmids, pMC3, pMC4, pMC5, pMC8 and pMC7, which contained 8bp oligonucleotide linkers in the Bam HI f region (figure 7).

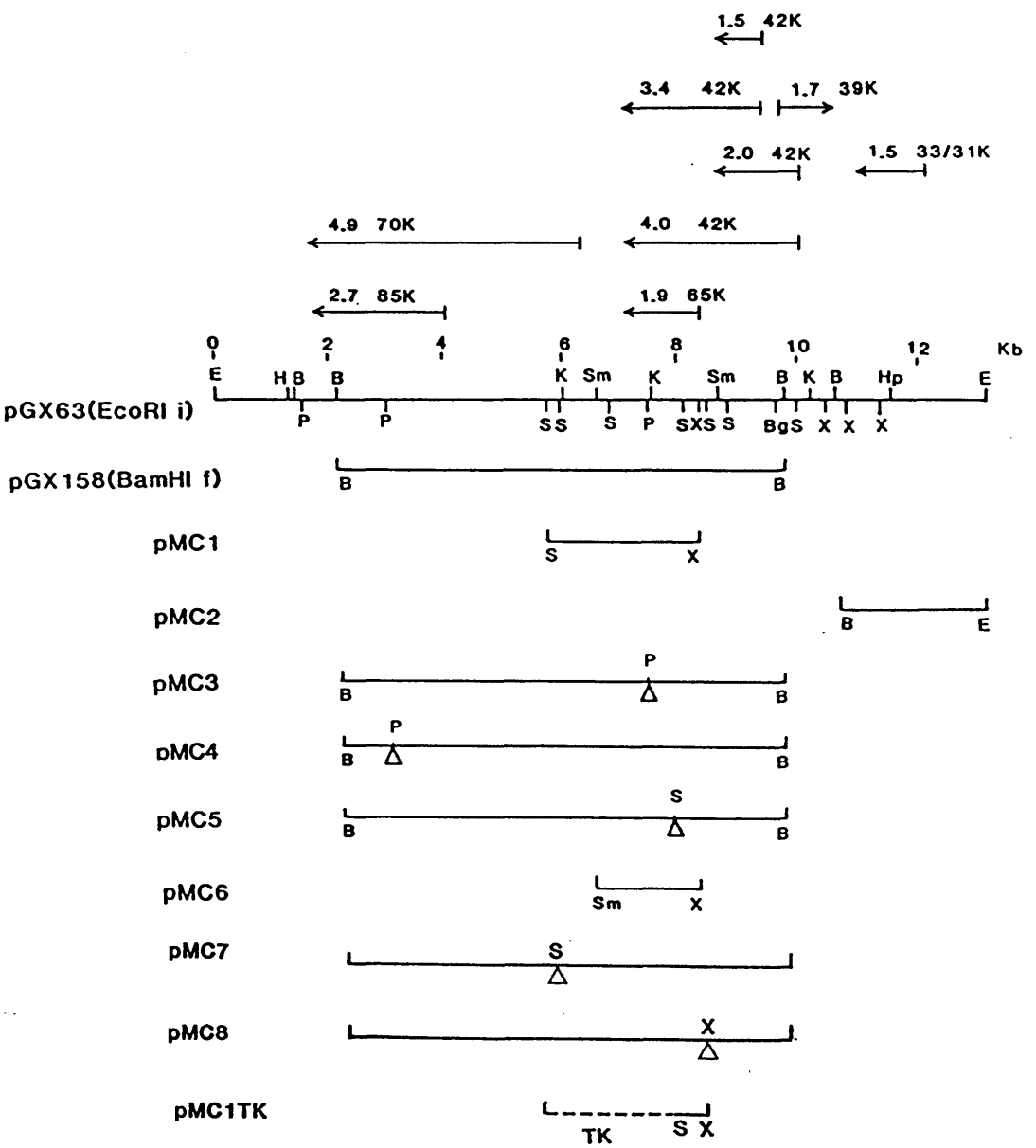

1.5 42K
3.4 42K
1.7 39K
2.0 42K
1.5 33/31K
4.9 70K
4.0 42K
2.7 85K
1.9 65K
0 2 4 6 8 10 12 Kb
E H B B K Sm K Sm B K B Hp E
P P S S S P S X S S BgS X X X
pGX63(EcoRI i)
pGX158(BamHI f)
B B
pMC1
S X
pMC2
B E
pMC3
P
B B
pMC4
P
B B
pMC5
S
B B
pMC6
Sm X
pMC7
S
pMC8
X
pMC1TK
TK S X

Figure 7. Subclones of pGX63 (EcoRI i)

A restriction map of the EcoRI i fragment is shown, together with mapping positions of the mRNAs, as desribed by Hall et al. (1982). The sizes of the mRNAs are in kb, including 0.2kb for the poly A tail, and polypeptide products (mw x 10^3 (K)) are indicated. Restriction endonuclease cleavage sites are abbreviated as follows: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; Hp, HpaI; K, KpnI; P, PvuII; S, SalI; Sm, SmaI; X, XhoI. Not all SalI and XhoI sites are shown. Construction of the plasmids is described below.

The BamHI f and BamHI/EcoRI sub-fragments were inserted into pAT153 to create pGX158 and pMC2, respectively. Plasmids pMC3 and pMC4 were prepared by partially digesting pGX158 with PvuII and the linear molecules were ligated with EcoRI 8bp linkers (GGAATTCC). For pMC5 and pMC7 a SalI site was converted to a StuI site by the insertion of an 8bp linker (CCTCGAGG). The linker was phosphorylated, ligated and cleaved with XhoI and was then inserted into pGX158, which was previously linearised by partial digestion with SalI. This manipulation altered the sequence at a SalI site from GTCGAC to GTCGAGGCCTCGAC, AGGCCT being a StuI site. Plasmid pMC8 was constructed by inserting cleaved XhoI linkers into the unique XhoI site pGX158. For the preparation of pMC1, a 2.6kb fragment of pMC7, delimited by the new StuI site and existing XhoI site was cloned between the SalI and SmaI sites of pUC9. Plasmid pMC6 was created by cloning the 2.4kb XhoI/SmaI HSV-1 fragment of PGX158 into pUC9. Plasmid pMC1TK was constructed by replacing the HSV-1 sequences between the unique SalI and EcoRI sites of pMC1 with a SalI/EcoRI fragment which contained the TK coding sequences. The TK-containing fragment was purified from pF1 (Pelham, 1982), which was kindly provided by Dr. H. Pelham.

Table 3. Stimulation of TK synthesis by cotransfection with cloned HSV-1 fragments.[a]

Fragment	Map co-ordinates	TK activity[b]	Stimulation[c]
None	-	54	-
KpnIb	0.02-0.11	55- 82	1.3 (3)[d]
EcoR Id	0.08-0.19	57- 57	1.1 (2)
BamHIa	0.14-0.23	53- 78	1.2 (2)
EcoR Ig	0.19-0.30	30- 68	0.8 (4)
EcoR If	0.31-0.42	22- 35	0.5 (2)
BamHIr	0.41-0.43	37- 43	0.7 (2)
EcoR Im	0.42-0.45	33- 37	0.7 (2)
KpnIc	0.44-0.52	45- 83	1.2 (3)
EcoR Ia	0.48-0.63	31- 63	0.9 (4)
KpnId	0.59-0.67	50- 64	1.1 (3)
EcoR Ii	0.63-0.72	118-262	3.9 (4)
KpnIo	0.70-0.73	51- 74	1.1 (4)
EcoR Ib	0.72-0.87	173-418	5.6 (8)
EcoR Ih	0.87-0.96	28- 41	0.6 (2)

a Cells were transfected with 0.5 ug of pTKN1 plus 1ug of test plasmid.

b TK activities are expressed as counts/min per min of assay per ug protein per ug plasmid. The range of observed values is given.

c Stimulation (fold) represents the mean increase in TK expression relative to the pTKN1 value.

d The number of separate determinations is shown in brackets.

A background of 5cpm/min/ug protein/ug plasmid was deducted from all values

Table 4. Stimulation of TK expression by two independantly isolated clones of EcoRI i.

Plasmid	TK activity	Stimulation
none	100	-
pGX69	838	8.4
pGX63	751	7.5

Experimental details and presentation of results are described in the legend to table 3.

Table 6. Stimulation of TK synthesis by linker insertion mutants of pGX158.

Plasmid	TK activity	Stimulation
pGX158	97-264	3.6 (4)
pMC3	30- 54	0.8 (2)
pMC4	151-284	4.0 (2)
pMC5	127-171	3.7 (2)
pMC7	139-275	3.8 (2)
pMC8	180-253	4.0 (3)
pGX158+pMC3	188-277	3.5 (2)

Presentation of results is described in the legend to table 3.

The insertion of one or two linkers would result in a disruption of the reading of an encoded polypeptide, thus preventing the expression of a functional polypeptide. The results of cotransfections utilising the linker insertion mutants (table 6) reveal that only the linker insertion of pMC3 negated the stimulatory response. The lack of activity was not due to an inhibitory effect of this plasmid as the positive regulatory effect of pGX158 was not reduced by addition of pMC3 during transfection.

The fact that activity was prevented by a frame shift mutation is indicative of the fact that an encoded polypeptide rather than an RNA molecule, per se was involved. According to the mapping data (figure 7), three transcripts of 1.7, 3.8 and 3.2kb are likely to be disrupted by the mutation in pMC3. However, insertions contained within pMC5 and pMC8, which interrupt the 3.8 and 3.2kb mRNAs were ineffective in preventing activity. It was therefore proposed that the 1.7kb mRNA encoded the trans-acting polypeptide.

6.5 Identification of sequences encoding the trans-acting polypeptide

In order to ensure that the 1.7kb mRNA alone was responsible for the increased expression from the IE regulatory sequences, pMC1 was constructed. This plasmid has a 2.6kb insert which contains the complete coding sequences only of the 1.7kb transcript. In cotransfection assays (table 5) pMC1 proved to be as effective as pGX158 (BamHI f) in stimulating TK expression. This evidence, together with data obtained in the linker insertion studies, strongly suggest that the IE gene trans-activator is encoded by the 1.7kb transcript which maps within pMC1.

6.6 Specificity of stimulation by EcoRI b and BamHI f

The results described in section 6.2 show that two distinct HSV-1 fragments are capable of stimulating TK expression when transfected with pTKN1.

An HSV-1 structural component stimulates transcription only when mediated by specific sequences

located upstream of IE genes (Mackem and Roizman, 1982a and b, Cordingley et al., 1983, Kristie and Roizman, 1984; Preston et al., 1984). In order to prove that one of the HSV fragments encoded the structural polypeptide involved, it was necessary to show that trans-activation by cotransfection with the genomic clone had the same target sequence requirement for IE upstream sequences as did induction of expression by superinfecting with UV inactivated virus. Plasmid pGX152 (EcoRI b) or pGX158 (BamHI f) was cotransfected with a series of hybrid plasmids which contained the TK gene under regulation of various promoters and upstream elements. Resultant TK expression was compared to that obtained when hybrid constructs were superinfected with UV inactivated tsK.

The chimaeric plasmids used in these experiments were already available and are depicted in figure 8. Plasmids pTKN2 and pTKN3SV1 (Cordingley et al., 1983) contain IE gene 3 5' sequences from -331 to +27 and -108 to +27, respectively. In addition, pTKN3SV1 contains a 270bp fragment of SV40 sequences located approximately 4Kb downstream from the 5' terminus of the TK mRNA. One copy of the 72bp repeat is contained within the SV40 sequences and the enhancer properties of this region boost expression of IE gene 3 promoter to an easily detectable level. Plasmids pS12TK and pS14TK (Preston et al.,1984) contain sequences from the IE gene 4/5 upstream region (IE genes 4 and 5 have an identical promoter sequence) from -379 to +114 and -315 to +114, respectively. The plasmid pSVTK1 is regulated by an SV40 early promoter and enhancer containing upstream region from -215 to +56.

In a cotransfection assay (table 7) TK expression of pTKN2 and pS12TK was stimulated by superinfection with UV inactivated tsK, in agreement with previous results (Cordingley et al., 1983). Increased TK activity was also observed when these plasmids were cotransfected with pGX158, which contains BamHI f. Infection with UV inactivated tsK or transfection with pGX158 did not stimulate expression of pTKN3SV1, pS14TK or pSVTK1. The control plasmid, pMC3 which contains a linker insertion in the BamHI f coding sequences

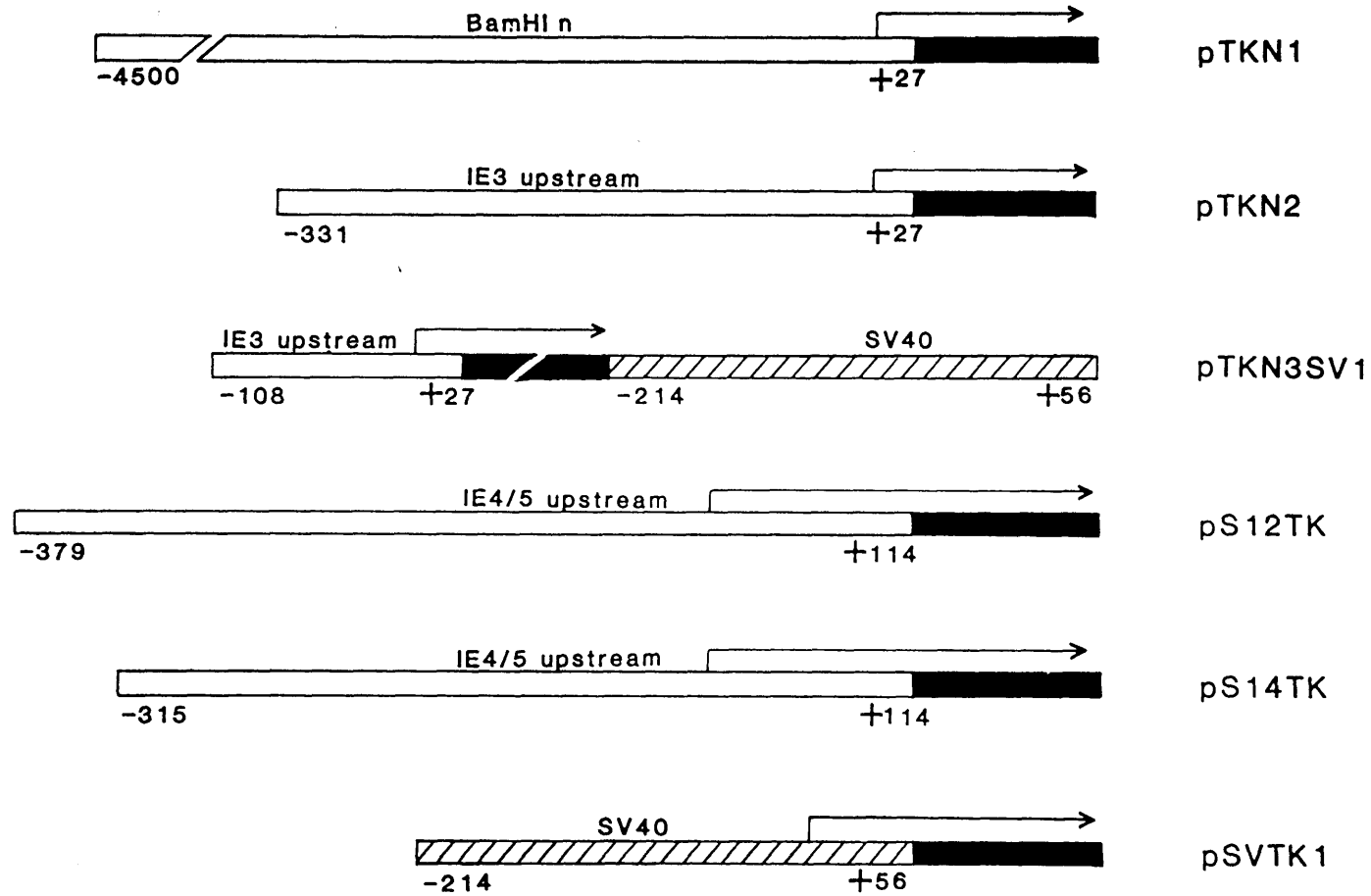

BamHI n
-4500
+27
pTKN1
IE3 upstream
-331
+27
pTKN2
IE3 upstream
SV40
-108
+27
-214
+56
pTKN3SV1
IE4/5 upstream
-379
+114
pS12TK
IE4/5 upstream
-315
+114
pS14TK
SV40
-214
+56
pSVTK1

Figure 8. Chimaeric TK plasmids.

Filled areas represent TK coding sequences, hatched areas denote SV40 sequences and open regions represent HSV-1 IE specific sequences derived from BamHI n. The co-ordinates shown refer to the usual start of IE mRNA 3 (pTKN2 and pTKN3SV1), IE mRNA 4/5 (pS12TK and pS14TK) or SV40 T antigen mRNA (pSVTK1).

Table 7. Stimulation of TK synthesis from chimaeric plasmids by cotransfection with pGX158, pMC3 or pGX152.

Chimaeric TK plasmid	Test plasmid/ superinfection	TK activity	Stimulation
pTKN2	none	100	-
pTKN2	pGX158	359-954	6.6 (4)
pTKN2	pMC3	100-118	1.1 (2)
pTKN2	pGX152	204-227	2.2 (3)
pTKN2	SI UV tsK	701-868	7.8 (2)
pTKN3SV1	none	5	
pTKN3SV1	pGX158	5- 5	1.0 (2)
pTKN3SV1	pMC3	3- 5	0.8 (8)
pTKN3SV1	pGX152	38- 47	8.5 (2)
pTKN3SV1	SI UV tsK	3- 5	0.8 (2)
pS12TK	none	62	
pS12TK	pGX158	190-419	5.0 (4)
pS12TK	pMC3	33- 47	0.6 (2)
pS12TK	pGX152	184-415	5.2 (3)
pS12TK	SI UV tsK	370-729	9.3 (3)
pS14TK	none	51	
pS14TK	pGX158	35- 85	1.3 (5)
pS14TK	pMC3	45- 55	1.0 (3)
pS14TK	pGX152	251-350	6.0 (3)
pS14TK	SI UV tsK	55- 72	1.3 (3)
pSVTK1	none	41	
pSVTK1	pGX158	28- 53	1.0 (3)
pSVTK1	pMC3	22- 24	0.6 (2)
pSVTK1	pGX152	105-236	4.1 (3)
pSVTK1	SI UV tsK	28- 57	1.1 (2)
none	SI UV tsK	ND	

Cells were transfected with 0.5ug of pTKN2, pS12TK, pS14TK, pSVTK1 or 1.0uq pTKN3SV1, plus 0.5ug of test plasmid. SI UV tsK: no test plasmid was added but cells were infected with UV irradiated tsK. Presentation of results is described in the legend to table 3. ND: not detectable above background.

was unable to elicit an effect from any of the plasmids tested. It was therefore concluded that the trans-activity of the pGX158 encoded product was specific to genes controlled by IE regulatory sequences and was likely to be the HSV-1 TIF. In contrast to the effects mediated by pGX158, increased TK activity was observed when pGX152 was cotransfected with any of the chimaeric plasmids. It would appear that one or more of the encoded products of the EcoRI b fragment is responsible for a general stimulation of expression. This effect is not specific to the TK gene under the control of IE upstream sequences. It is therefore unlikely that EcoRI b encodes a component of the TIF.

6.7 Identification of the polypeptides encoded by pMC1

Analysis of intertypic recombinants identified a polypeptide, Vmw65/64, which maps between co-ordinates 0.65 to 0.69 in the HSV-1 genome (Marsden et al., 1976, 1978), a region which spans the HSV-1 sequences of pMC1. This polypeptide, now referred to as Vmw65, is believed to be equivalent to VP 16, which was described by Roizman and Furlong (1974) as a major tegument polypeptide. Hall et al. (1982) found the only detectable mRNA species to map within pMC1, a 1.7 kb species, to encode a product of approximate mw 65000, when synthesised in in vitro translation systems. It therefore appeared probable that Vmw65 was encoded by pMC1. In order to test this theory the in vitro translation of infected cell RNA was arrested by hybridisation with sequences from this region of the genome.

Total cytoplasmic RNA was hybridised with pMC6 (a sub-clone of pMC1, figure 7) prior to its translation in an in vitro system. Control RNA samples, which had been hybridised with vector (pUC9) DNA, or without DNA were also translated. As a further control identical samples were denatured prior to in vitro synthesis of the polypeptides. Half of each sample of translated products was retained for immunoprecipitation analysis. The remainder was electrophoresed on an SDS polyacrylamide gel (figure 9), which shows that efficient translation of all samples

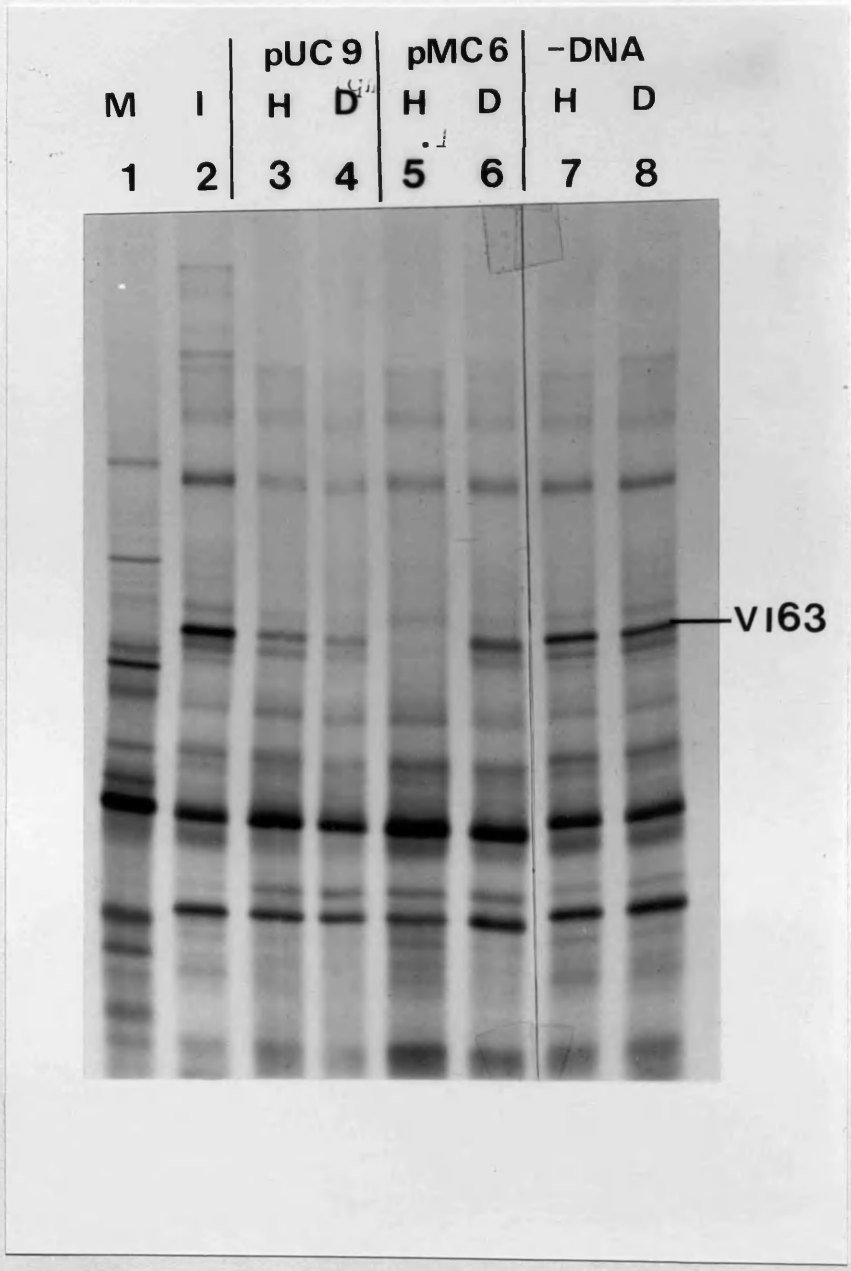

pUC 9
pMC 6
-DNA
M
I
H
D
H
D
H
D
1
2
3
4
5
6
7
8
VI63

Figure 9. Hybrid arrested in vitro translated products.

HSV-1-infected cell RNA was hybridized (H) or hybridized and subsequently denatured (D) with pUC9 (tracks 3 and 4), with pMC6 (tracks 5 and 6) and without DNA (tracks 7 and 8). Mock-infected RNA (track 1) and infected-cell RNA (track 2) translated without further treatment. Samples were electrophoresed on a 10% SDS polyacrylamide gel.

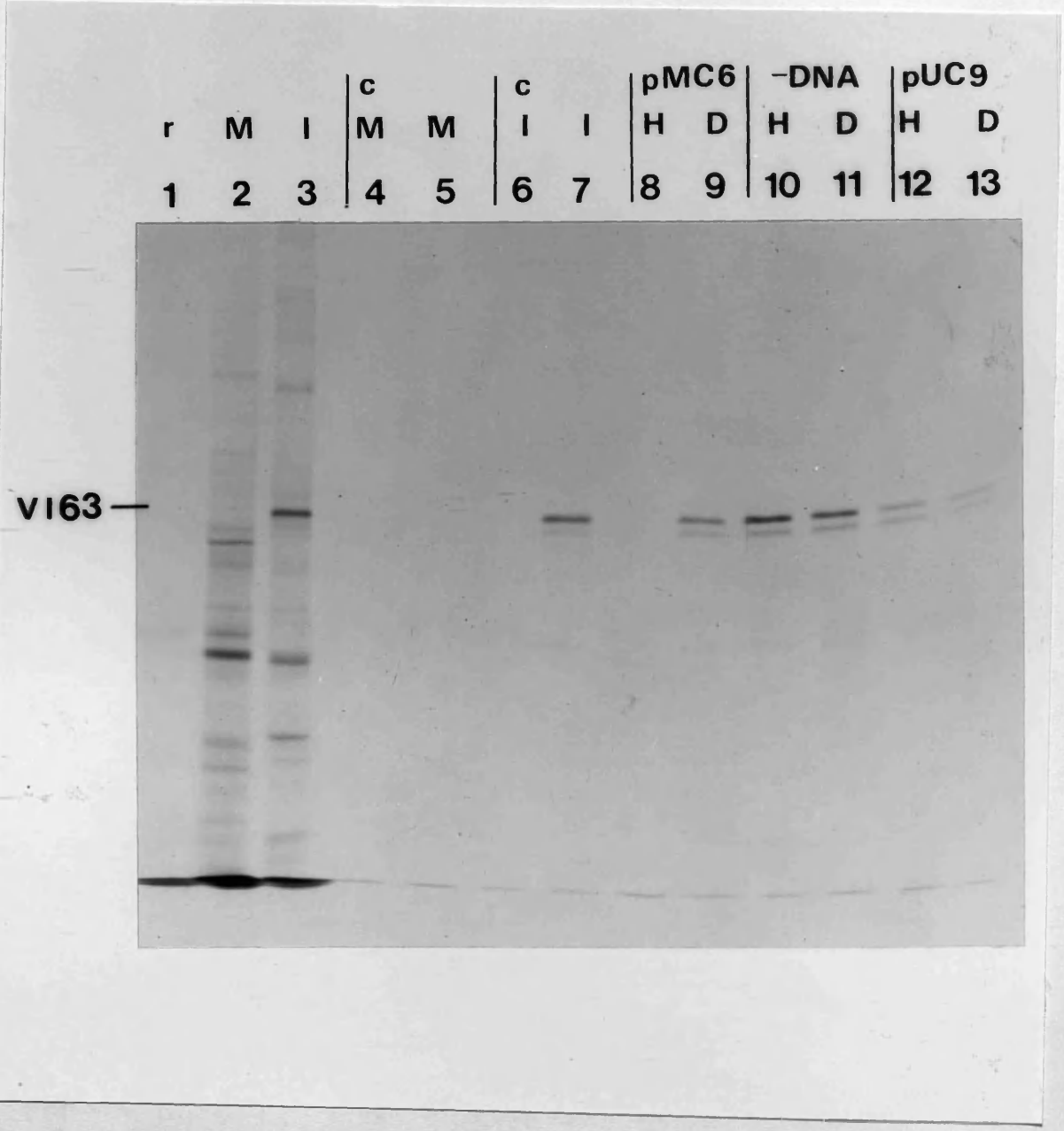

r M I c M M c I I pMC6 H D -DNA H D pUC9 H D
1 2 3 4 5 6 7 8 9 10 11 12 13
VI63

Figure 10. Immunoprecipitates of hybrid arrested in vitro translated samples.

Total in vitro translation of E. coli rRNA (track 1), mock infected BHK RNA (track 2) and HSV-1 infected cell RNA (track 3). Translated products were immunoprecipitated with MA1044 (tracks 5 and 7-13) or control ascites fluid (tracks 4 and 6). Immunoprecipitation of mock infected RNA (tracks 4 and 5) or infected cell RNA (tracks 6-13) translation products. The infected cell RNA was hybridised (H) or hybridized and denatured (D) with pMC6 (tracks 8 and 9), without DNA (tracks 10 and 11) and with pUC9 (tracks 12 and 13), prior to translation and immunoprecipitation. Samples were electrophoresed on a 10% polyacrylamide gel.

occurred. The polypeptides depicted in track 5 are derived from mRNA which was hybridised to pMC6 before translation. Species VI63 (described by Preston, 1977) and VI61 were absent, indicating that these polypeptides are specified by the mRNA to which pMC6 hybridises. This result is compatible with that obtained by Hall et al. (1982), who describe the translation product of the 1.7 kb mRNA as a polypeptide of molecular weight 65,000.

In vitro translated samples were reacted with monoclonal antibody MA1044, known to be directed against the major tegument species, Vmw65 (JW Palfreyman, personal communication). Only polypeptides VI63 and VI61 were found to be immunoprecipitated (figure 10). Neither product was precipitated when hybridised to pMC6 prior to translation (track 8), although other polypeptide species were efficiently synthesised (figure 9, track 5). Denaturation of the sample prior to translation permitted precipitable products to be synthesised (figure 10, track 9), showing their absence to be due to hybridisation with pMC6. Hybridisation with pUC9 did not arrest the production of VI61 and VI63 (tracks 12 and 13) and they were not synthesised from mock infected RNA (track 2 and 5).

These results demonstrate that the 1.7kb mRNA which maps in pMC1 (Hall et al., 1982; Dalrymple et al., 1985; Pellet et al., 1985) encodes the major structural polypeptide, Vmw65.

6.8 Promoter efficiency of the gene encoding Vmw65

The gene encoding Vmw65 is transcribed maximally at late times of infection and is referred to as a γ_1 (late) species (Hall et al., 1982). Late genes require functional Vmw175 in order to be transcribed during the lytic cycle (Watson and Clements , 1980).

In order to ensure that Vmw65 would be expressed in transient assays, the promoter of the encoding gene was tested for its ability to function in transfected cells.

The experiments described in section 6.4 show that the insertional mutation of pMC5 did not destroy trans-activating potential. It was therefore assumed that

Table 8. TK synthesis in transfected cells

Plasmid	TK activity[a]
pTK1 (0.5ug)	ND[b]
pTK1 (3.0ug)	23
pMC1TK (0.5ug)	8
pMC1TK (3.0ug)	73
pTKN2 (0.5ug)	52

a TK activities are expressed as counts/min per min of assay per ug protein. The values given are the means of 3 determinations. A background of 6 cts/min of assay per ug protein has been subtracted from all values.

b ND, not detectable above background.

the SalI site into which the linker was inserted was upstream of essential coding sequences. A hybrid plasmid, pMClTK, was constructed in which the TK gene was placed under the regulation of the Vmw65 promoter (figure 7). Subsequent sequence data has, however, revealed that pMClTK contained the 5' end of the Vmw65 coding sequences (Dalrymple et al., 1985). Fortunately the Vmw65 initiation codon is in phase with that of the TK gene and an active enzyme is synthesised from pMClTK.

TK expression of pMClTK was compared to that of pTKN2, which consists of the TK gene under the control of the promoter and upstream region of IE gene 3 (figure 8) and also to pTKl which has the TK gene under control of its natural 5' flanking sequences. When 3ug of pMClTK was transfected into BHK cells, TK activity was found to be three times as great as that of cells transfected with pTKl (table 8). Plasmid pMClTK, was found, however, to be six fold less active than pTKN2. These results demonstrate that the gene encoding Vmw65 possesses an active promoter and is efficiently expressed in BHK cells. Therefore Vmw65 would be available in transfected cells to mediate the stimulation of IE genes.

6.9 Discussion

The data presented in section 6.2 identify DNA fragments from two regions of the HSV-1 genome, EcoR I i (0.62 to 0.72mu) and EcoRi b (0.72 to 0.87mu) which stimulate TK expression from cotransfected plasmids in BHK cells.

6.9.1 EcoRI i The activity of the EcoR I i fragment was retained in a subfragment, BamHI f (0.64 to 0.69mu). Stimulation of TK expression by the BamHI f plasmid, pGX158, was only observed when the TK gene of the cotransfected chimaera was placed under regulation of the HSV IE gene upstream sequences required to mediate a response to the TIF when delivered by superinfecting, UV inactivated virus. IE gene 3 sequences necessary for activation by cotransfection or by superinfection were located between -331 and -108, while requisite IE gene 4/5 sequences were situated between

-379 and -315 from the mRNA initiation site. These results were in agreement with previous mapping data concerning the sequences which respond to the TIF (Mackem and Roizman, 1982a, b, Cordingley et al., 1983, Preston et al., 1984). Both of the IE upstream regions contained cis-acting elements relating to the consensus.

Previous studies had shown that stimulation of IE gene expression, mediated by the TIF, was due to to an increase in transcriptional activity (Preston et al., 1984, Lang et al., 1984). Experiments carried out by Dr C.M. Preston confirmed that trans-activation by pGX158 was the result of increased accumulation of correctly initiated mRNA transcripts (Campbell et al., 1984). The increase was believed to be due to an elevated rate of transcription rather than increased stability of the mRNAs as pS12TK, but not pS14TK, was responsive. Both of the plasmids (figure 8) encode identical transcripts and differ only in the extent of the IE upstream, nontranscribed sequences.

The stimulatory sequences within the EcoRI f fragment were localised to the 2.6kb region (0.669 to 0.685mu) contained within pMC1. According to the mapping data of Hall et al.(1982) only one mRNA of 1.7kb (excluding the poly A tail) was encoded entirely within this region. Subsequent S1 analysis confirmed this result and accurately positioned the 5' and 3' termini of the transcript at 274bp and 589bp, respectively, from the ends of the pMC1 HSV-1 insert (Dalrymple et al., 1985; Pellett et al., 1985). The insertion of an 8bp linker in the region of pGX158 which specified the 1.7kb transcript abolished stimulatory ability, verifying that an encoded polypeptide was responsible. It was predicted that such an insertional mutation would result in the out of phase translation of 60% of the polypeptide.

The nucleotide sequence of the pMC1 fragment has now been determined for two HSV-1 strains (17 and F) (Dalrymple et al., 1985; Pellett et al., 1985). Only one open reading frame of significant length was recognised within these sequences which correlated with mapping data of the 1.7kb transcript, arguing against the possibility that the IE specific

stimulation was due to a minor species sharing coding sequences with the 1.7 kb mRNA. Further proof comes from the finding that 12bp linker insertions in the 5' coding sequences do not prevent *trans*-activation (C.I. Ace, personal communication).

Hybrid arrest of *in vitro* translation with pMC1, a sub clone of pMC1, followed by immunoprecipitation with monoclonal antibody revealed that pMC1 encoded Vmw65 (designated VP16 in alternative nomenclature), a major structural phosphoprotein located in the tegument of the virion (Roizman and Furlong, 1974; Marsden *et al*., 1978; Lemaster and Roizman, 1980; Johnston *et al*., 1984). The identification of the TIF as a tegument polypeptide is consistent with studies of Batterson and Roizman (1984) who located the stimulatory factor outside the nucleocapsid. The transfection studies also showed that Vmw65 alone is capable of the stimulation of transcription and the integrity of the tegument does not require to be preserved in order for *trans*-activation to occur. However, McKnight *et al*. (1987) found that the products of two open reading frames located 3' to the gene encoding Vmw65 affected its function. One gene product (of UL 46) enhanced *trans*-inducing activity, while the other (of UL 47) caused a reduction in Vmw65 activated IE gene expression.

Two potential translation initiation sites are observed in the 5' domain of the Vmw65 coding sequences, both of which are in phase (Dalrymple *et al*., 1985, Pellett *et al*., 1985). The 5' site apears to be the most frequently utilised and initiation from this codon is likely to result in the synthesis of the *in vitro* translation product VI63, while the second site presumably directs translation of the minor species VI61 (figures 9 and 10). The smaller polypeptide is fully active, as the introduction of an 8bp linker between the first and second initiation codons, to create pMC5, failed to prevent the *trans*-activating potential of pGX158. Differentially sized polypeptides are also found to result from the utilisation of in phase initiation codons of the TK gene (Marsden *et al*., 1983).

The IE *trans*-activating properties of plasmids

encoding Vmw 65 have now been confirmed by a number of workers (O'Hare and Hayward, 1985b; Pellett et al., 1985; Bzik and Preston, 1986; Gelman and Silverstein, 1986) and have been utilised to perform a detailed analysis of the IE gene 3 promoter (Bzik and Preston, 1986). O'Hare and Hayward (1985b) have found the HSV-2 TIF to map between 0.648 and 0.695mu.

Vmw65 is efficiently expressed and is a major product of in vitro translation (Hall et al., 1982; this study, figure 9) and of lytic infection (Heine et al., 1974,; Marsden et al., 1976). In the studies presented it was shown that the gene encoding Vmw65 possesses an efficient promoter. Despite the fact that functional Vmw175 is continually required throughout the lytic cycle to allow transcription of early and late genes, the Vmw65 promoter is active in transfected cells in the absence of IE gene products. As discussed in section 2.2.4 a number of other early and late species are found to be detectably expressed in uninfected systems. Vmw65 is maximally expressed at late times of infection and is described as a γ_1 (late) species (Hall et al., 1982).

Nucleotide sequence data reveals the presence of motifs recognised as transcriptional control signals in the 5' flancing sequence of the gene encoding Vmw65. A TATA box was identified at -30, a CCAAT box at -78 and a probable Sp1 binding site at -48 and -107 (Dalrymple et al., 1985; Pellett et al., 1985). These elements were found to be involved in the transcriptional regulation of the early genes, TK and gD (McKnight and Kingsbury, 1982; McKnight et al., 1981) and their role in eukaryotic transcription was discussed in section 3.1 and 3.2.

6.9.2 EcoRI b

The plasmid pGX152 containing the HSV-1 EcoRI b fragment was found to stimulate expression of plasmids controlled by IE promoters plus upstream regulatory sequences. Expression of pSVTK1, regulated by the SV40

promoter and enhancer, and pTKN3SV1, regulated by the IE gene 3 promoter without the upstream region was also increased by cotransfection with pGX152. The *trans* acting effect of PGX152 was therefore not specific to IE transcription as the upstream TAATGARATTC was not essential to mediate a response. The EcoRI b fragment contains sequences from IR_S, IR_L and U_L and encodes IE genes 1, 2 and 3, in addition to other genes of unknown function (figure 11). As discussed in section 2.3.1 the products of these IE genes are known to be *trans*-activators of early and late gene expression and cotransfection with plasmids encoding the IE polypeptides, Vmw175 and Vmw110, either singly or together, can modulate IE gene expression. Far upstream IE sequences are not required to mediate this control (O'Hare and Hayward, 1987; Gelman and Silverstein, 1986). The *trans*-activating effect of pGX152 was therefore believed to be due to one or more of the encoded IE polypeptides.

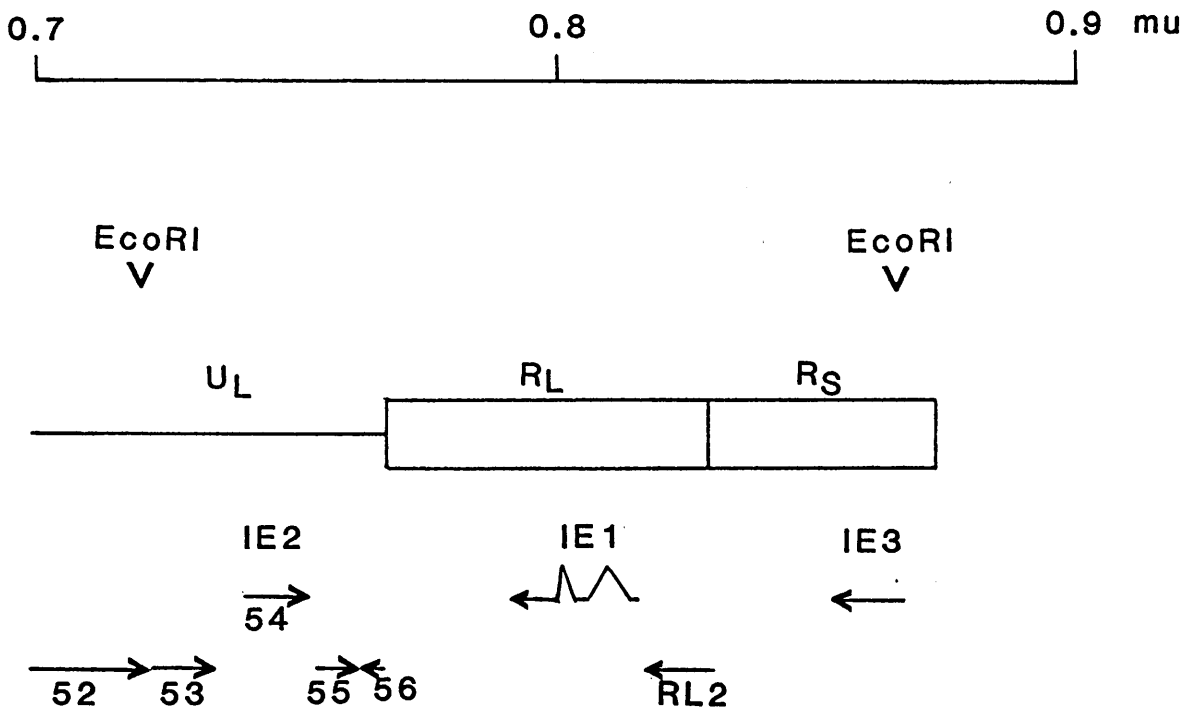

0.7
0.8
0.9 mu
EcoRI
EcoRI
U_L
R_L
R_S
IE2
IE1
IE3
54
52
53
55
56
RL2

Figure 11. Map positions of genes within EcoRI b.

Genes 52-56 have been positioned by analysis of open reading frames within the DNA sequence and are numbered from the left hand end of U_L (Perry, 1986; D.J. McGeoch personal communication). The positions of the mRNAs within R_L and R_S (plus IE 2) have been determined by S1 nuclease mapping in addition to analysis of DNA sequence data (Mackem and Roizman, 1982b; Rixon et al., 1983; Chou and Roizman, 1986; McGeoch et al., 1986).

7 REGULATION OF EXPRESSION OF THE MAJOR IE GENE OF PSEUDORABIES VIRUS

7.1 Introduction

Pseudorabies virus is related to HSV-1 on the basis of morphological and biological properties and both viruses are members of the subfamily alphaherpesvirinae (Sabin, 1934; Roizman, 1982). Homology between the genomes of HSV and PRV has been estimated to be approximately 8% by cross-hybridisation experiments (Bronson et al., 1972; Ludwig et al., 1972; Rand and Ben-Porat, 1980). Genome arrangement is different, as PRV lacks inverted repeat sequences corresponding to those which bracket the long unique region of HSV (figure 2)(Sheldrick and Berthelot, 1974; Stevely, 1977). Despite the apparent low level of sequence homology, the PRV and HSV genomes have a partial colinear arrangement of genes encoding similar functions (Ben-Porat et al., 1983b; Davison and Wilkie, 1983b). HSV has five IE genes while PRV has only one major IE gene which shares sequence homology with IE gene 3 of HSV-1 (Ben-Porat et al., 1983b; Davison and Wilkie, 1983b). The product of HSV-1 IE gene 3 (Vmw175), and the PRV IEP (estimated mw 180,000) are known to be required to initiate and maintain the early and late phases of viral gene expression (Preston, 1979a; Watson and Clements, 1980; Ihara et al., 1983).

The PRV IE gene is efficiently expressed only at very early times, or during infection with the mutant, tsG, which has a defect in the IEP (Feldman et al., 1982), suggesting that, like HSV-1, induction of IE gene transcription may be effected by a trans-acting factor. Previous studies, however, have shown that infection with PRV does not stimulate expression from genes under the control of HSV IE gene 3 upstream sequences (Batterson and Roizman, 1983). The most likely explanation of this result is that PRV particles possess a component only able to regulate the expression of PRV IE genes, thus indicating a specificity in the interaction between the IE upstream sequence and trans-acting virion factor.

The regulation of the PRV IE gene was therefore

investigated, with two main aims. The first was to elucidate the mechanisms of transcriptional regulation in PRV, and the second to investigate the relevance of trans-acting factors in IE gene control of herpesviruses in general. This section describes a comparison of the regulation of the PRV IE gene to that of HSV-1 IE gene 3, by the construction of chimaeric plasmids in which the HSV TK structural gene was placed under the control of the PRV IE gene upstream sequences. The regulation of these chimaeric genes was then analysed in transient expression assays.

7.2 Mapping the 5' terminus of the PRV IE transcript

In order to investigate the regulation of the PRV IE gene it was necessary to accurately locate the 5' end of the transcript. Previous mapping experiments using the Southern blotting technique have shown that the PRV IE gene is located internally within the inverted repeats and is therefore present in two copies per genome (Feldman et al., 1979) (figure 12A). The orientation of the IE transcripts has been determined by hybridisation of cDNA complementary to the mRNA 3' terminus to restriction endonuclease fragments of viral DNA (F.J. Rixon, personal communication) and the 5' end of the terminal copy is known to map within the KpnI h fragment (Feldman et al., 1979; Ihara et al., 1983). Everett (1984b) successfully utilised cloned KpnI h fragment to express the functional product in tissue culture, showing this fragment to contain all the necessary coding and regulatory sequences. Plasmid KpnI h was therefore used to study the sequences involved in PRV IE gene control.

Nuclease S1 mapping was used to accurately position the 5' end of the IE transcript. Immediate early RNA was prepared from cells infected in the presence of cycloheximide. Two independent probes were used, 5' labelled at an NcoI site or at a BamHI site, as shown in figure 12B. Following denaturation the probes were hybridised to PRV IE RNA, prepared from cells infected in the presence of cycloheximide, and to mock infected cell RNA. The probe labelled at the NcoI site gave rise to a 270bp fragment (figure 13, NcoI, IE track), while the probe

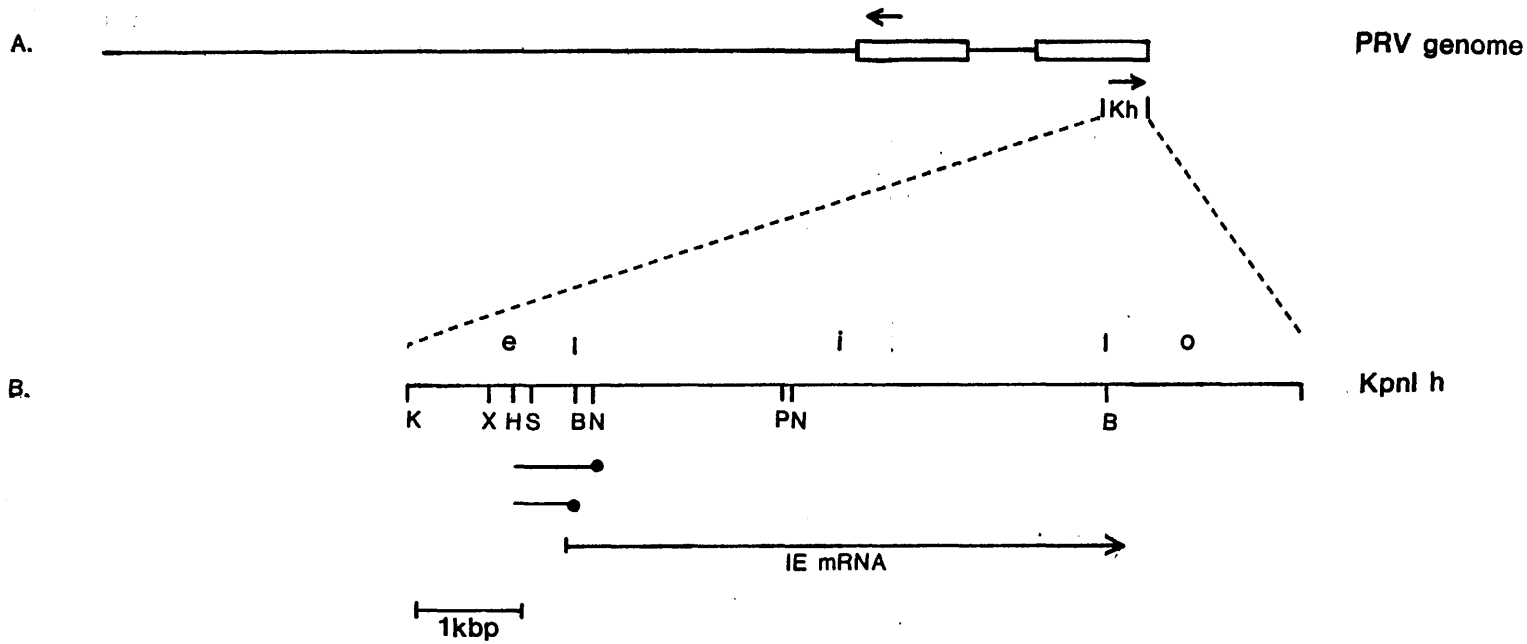

A.
PRV genome
Kh
e
I
i
I
o
B.
KpnI h
K
X
H
S
BN
PN
B
IE mRNA
1kbp

Figure 12. A. PRV genome.

Locations and orientations of the PRV IE transcript are shown.

B. Restriction map of the KpnI h fragment.

Abbreviations are as follows: K, KpnI; X, XhoI; H, HindIII; S, SmaI; B, BamHI; P, PstI; N, NcoI. Only relevant XhoI, PstI and SmaI sites are shown. The nomenclature of the BamHI fragments is indicated above the map and the 5' labelled probes used in S1 mapping are shown below. The location of the major mRNA is shown.

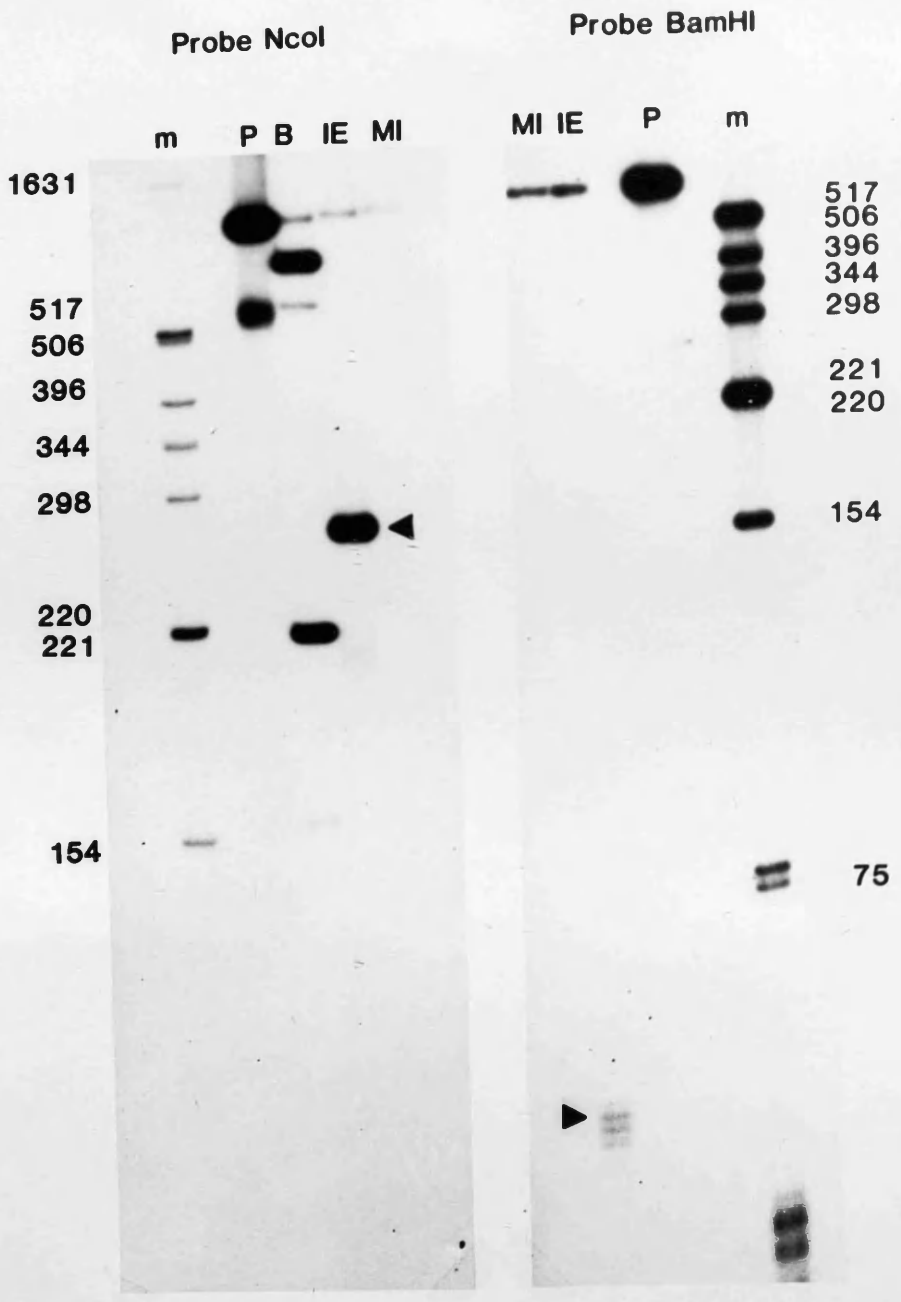

Probe NcoI
m
P
B
IE
MI
1631
517
506
396
344
298
220
221
154
Probe BamHI
MI
IE
P
m
517
506
396
344
298
221
220
154
75

Figure 13. S1 nuclease mapping to determine 5' end of the PRV IE mRNA.

The probes used are indicated above panels. Left and right panels show 8% and 10% denaturing gels respectively. Tracks are labelled as follows: m, markers (HinfI digested pBR322); P, probe (as indicated); B, BamHI-digested probe; IE, probe hybridised to PRV IE cytoplasmic RNA; MI, probe hybridised to mock infected cell RNA. The hybrid bands are indicated by arrows and are discussed in the text.

labelled at the BamHI site resulted in a 50bp fragment (figure 13 BamHI, IE track). No protected species were observed when the probes were hybridised to mock infected cell RNA. The sizes of the hybrids are consistent since the distance between the BamHI and NcoI sites is estimated as 220bp by NcoI digestion of fragment labelled at the BamHI site (NcoI, track B), and they locate the mRNA 5' terminus at approximately 50bp from the BamHI site between fragments e and i.

The 3' end of the PRV IE RNA has been mapped by nuclease S1 analysis (F.J. Rixon, personal communication) and is found to be approximately 250bp downstream of the BamHI site at the junction of the i and o fragments. Based on this information the size of the PRV IE transcript is 5.1kb (figure 12B).

7.3 Transient expression assays using PRV-TK hybrid plasmids

A series of hybrid plasmids was constructed in which the HSV TK coding sequences were placed downstream of various extents of PRV IE upstream regions as shown in figure 14. In these constructs the expression of the TK gene is regulated by the upstream PRV sequences. This strategy had been employed previously to determine the sequence requirements for HSV IE gene activation (Post *et al*, 1981; Mackem and Roizman, 1982a; Cordingley *et al*, 1983).

The stimulatory properties of the PRV hybrid plasmids were compared to those of pTKN2 and pTKN3SV2 (also shown in figure 14) using transient expression assays. Plasmid pTKN2 contains all necessary HSV-1 IE upstream sequences for *trans*-activation by the TIF (Mackem and Roizman, 1982a; Cordingley *et al*., 1983), whereas pTKN3SV2 contains only the promoter element (up to position -108) of IE gene 3 and therefore does not respond to the TIF since it lacks the IE gene 3 upstream region (section 6.6, Campbell *et al*., 1984). Expression from the IE gene 3 promoter in pTKN3SV2 is increased to a detectable level by the presence of an SV40 enhancer and would be expected to remain unaffected by

superinfection.

The plasmids were transfected into BHK cells and superinfections were carried out using UV-irradiated samples of tsK, a temperature sensitive mutant in IE gene 3 of HSV-1, or UV-irradiated PRV. Alternatively, cells were cotransfected with chimaeric plasmid plus pMC1, which encodes the HSV-1 TIF, Vmw65 (section 6, Campbell et al., 1984). The results (table 9) show that superinfection with 300 particles of UV-irradiated PRV did not stimulate TK expression from any of the plasmids tested. In contrast, superinfection with an equal number of UV-treated tsK particles or cotransfection with pMC1 elevated expression from pTKPRV1, pTKPRV2, pTKPRV3 and pTKN2 by 5 to 6 fold. In addition, experiments were carried out in which the monolayers were infected using 3000 PRV particles per cell (table 10). Again, no increase in expression was detected. The level of TK activity in cells transfected with pTKN3SV2 was unaffected by superinfection with PRV or tsK, showing that there was no inhibitory effect on cellular macromolecular synthesis, caused by superinfection, which could nullify a positive response. From these results it is concluded that PRV particles are unable to increase expression from either HSV-1 or PRV IE upstream sequences and therefore PRV lacks an effective TIF. PRV, however, contains IE upstream sequences which mediate a response to the HSV-1 TIF, whether delivered by infection with UV irradiated tsK or by transfection with pMC1.

In the absence of induction by the HSV-1 TIF, expression from pTKPRV1, pTKPRV2 and pTKPRV3 was as efficient as that of pTKN2, which contains the HSV-1 IE gene 3 enhancer-like element (Lang et al., 1984; Preston and Tannahill, 1984). It is probable, therefore, that the PRV IE upstream region possesses a very efficient promoter or has an element with enhancer properties.

In addition to using BHK cells, transfections were also carried out using pig kidney cells, since the pig is a natural host of PRV. In PK cells (table 11) infection with tsk also stimulated expression from PRV or HSV IE upstream sequences more effectively than PRV, suggesting that the

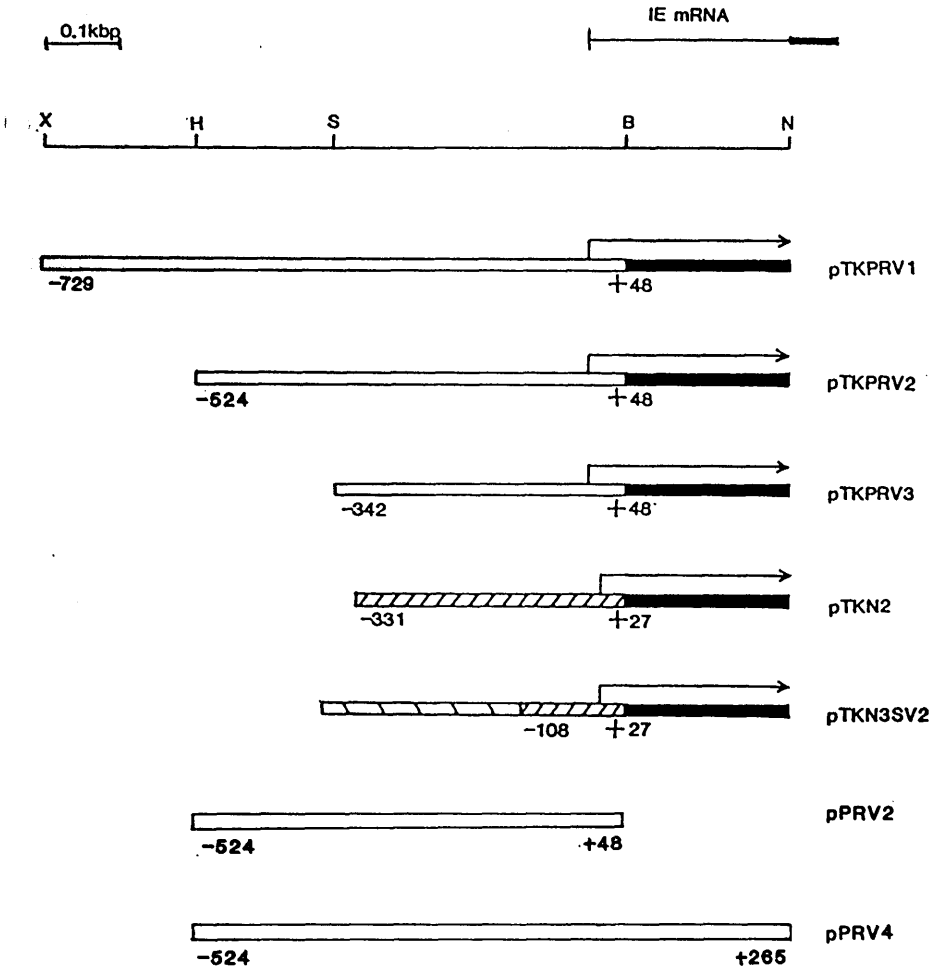

0.1kbp
IE mRNA
X
H
S
B
N
-729
+48
pTKPRV1
-524
+48
pTKPRV2
-342
+48
pTKPRV3
-331
+27
pTKN2
-108
+27
pTKN3SV2
-524
+48
pPRV2
-524
+265
pPRV4

Figure 14. PRV plasmids.

Origin of sequences is as follows: solid shading, structural TK gene; open areas, upstream sequences of the PRV IE gene (as defined by S1 nuclease mapping); hatched areas (/////), HSV-1 IE gene 3 upstream sequences; hatched area (\ \ \), SV40 sequences. Arrows and co-ordinates refer to the usual start of the mRNA. Plasmids pTKPRV1 and pTKPRV2 consist of 777bp XhoI/BamHI and 572bp HindIII/BamHI fragments of KpnI h, respectively, linked to the BglII/EcoRI fragment which contains the HSV-1 TK gene coding sequences (Wilkie et al., 1979). Plasmid pTKPRV3 consists of a 383bp SmaI/BamHI fragment of KpnIh linked to the BglII/BamHI insert which contains the TK gene. The vector used in all cases was pUC9 (Vieira and Messing, 1982). Plasmids pTKN2 and pTKN3SV2 have been described previously (section 6.6; Campbell et al., 1984).

Table 9. Transient expression in BHK cells

Chimaeric TK plasmid[b]	TK activity[a] Superinfecting virus or cotransfecting plasmid[c]			
	Mock infected	UV PRV	UV tsK	pMC1
pTKPRV1	108±27[d](6)[e]	103±27(5)	625±139(5)	627±128(5)
pTKPRV2	92±23 (6)	93±31(5)	544±93 (5)	518±174(4)
pTKPRV3	119±30 (4)	98±17(3)	601±61 (3)	637±85 (3)
pTKN2	83 (6)	96±22(5)	478±64 (4)	480±47 (5)
pTKN3SV2	58±14 (2)	61±13(2)	59±13 (2)	not done
None	0[f]	ND[g](2)	ND (2)	not done

a TK activity is expressed as counts per min per ug of protein per min of assay per ug chimaeric plasmid after normalisation to the pTKN2 value.

b Cells were transfected with 0.6ug of chimaeric plasmid plus lug of pMC1, where applicable.

c Superinfections were carried out using 300 particles per cell of UV-irradiated PRV or tsK per cell.

d Standard error.

e Number of independent determinations is shown in brackets.

f The actual value of 6 was subtracted from all the values as a background.

g Not detectable above background.

Table 10. Superinfection of pTKPRV2 transfected cells with various amounts of UV irradiated PRV.

number of UV PRV particles/cell	TK activity
0	92
10	93
30	81
100	117
300	93
1000	87
3000	95
(+pMCl) 0	597

Transfection conditions and presentation of results are described in table 9.

Table 11. Transient expression in PK cells.

Chimaeric TK plasmid	TK activity SI virus or cotransfecting plasmid		
	Mock infected	UV PRV	UV tsk
pTKPRV2	4	9	26
pTKN2	ND	ND	8

Transfection conditions and presentation of results are as described in table 9, except that PK15 cells are used instead of BHK cells.

A background of 6cpm/min/ug protein/ug plasmid was deducted from all values.

absence of a species specific factor did not prevent full activation by a PRV trans-acting polypeptide in BHK cells. The small (two fold) increase caused by PRV in PK cells is probably insignificant in view of the low levels of expression in this cell type.

7.4 Comparison of viral and plasmid DNA

It was important to ensure that the failure of the pTKPRV plasmids to respond to superinfection by UV-irradiated PRV was not caused by deletion of sequences during cloning. This was considered to be possible since an origin of replication has been located within the repeat region of the genome (Jean et al., 1977) and deletions have been found to occur when some replication origins are cloned, as in the case of the HSV-1 U_L origin (Spaete and Frenkel, 1982). The plasmid pKpnIh was therefore analysed to ensure that deletion of upstream sequences had not occurred. A restriction digest of plasmid DNA and of PRV genomic DNA was electrophoresed and transferred to nitrocellulose using the Southern blotting technique. The membrane was then probed with plasmid pPRV2, which contains the BamHI/HindIII fragment from -524 to +48 of the IE gene (figure 14). The cloned PRV band to which the probe hybridized had migrated the same distance (figure 15, pKpnIh track) as the equivalent genomic DNA fragment (PRV track), showing that no detectable deletion had occurred.

7.5 Co-infection with PRV and HSV-1

The experiments carried out in section 7.3 reveal that the HSV-1 TIF is able to induce expression of the TK gene under the control of the PRV IE upstream sequences. In order to determine whether HSV-1 could increase expression of the PRV IE gene in the viral genome BHK cells were coinfected with the two viruses. The cells were infected in the presence of cycloheximide, allowing only the IE mRNAs of both viruses to be synthesised. After incubation for 4h at 37°C the cycloheximide was removed and the plates were washed. Actinomycin D was added to prevent transcription of other mRNA species, while the absence of cycloheximide

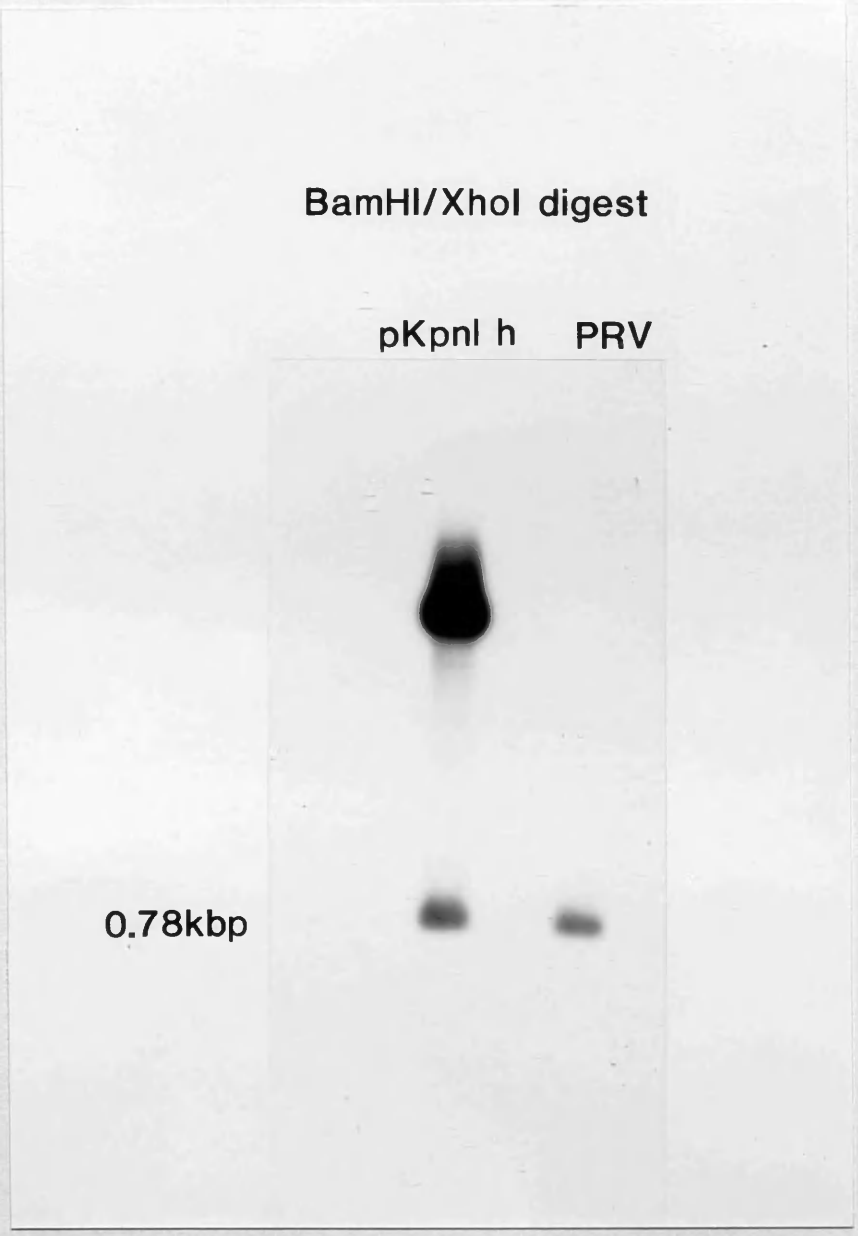

BamHI/XhoI digest
pKpnI h
PRV
0.78kbp

Figure 15. Comparison of cloned (pKpnIh) and genomic (PRV) DNA.

Plasmid pKpnIh or PRV DNA was cleaved with BamHI plus XhoI, and a blot of the digests was probed with pPRV2 which contains the BamHI/HindIII fragment (as in pTKPRV2, Fig.3). The equivalent 0.78kbp band is indicated. The larger band in the pKpnIh track is due to the hybridisation of pUC9 DNA in the probe to pAT153 sequences in the restricted plasmid DNA.

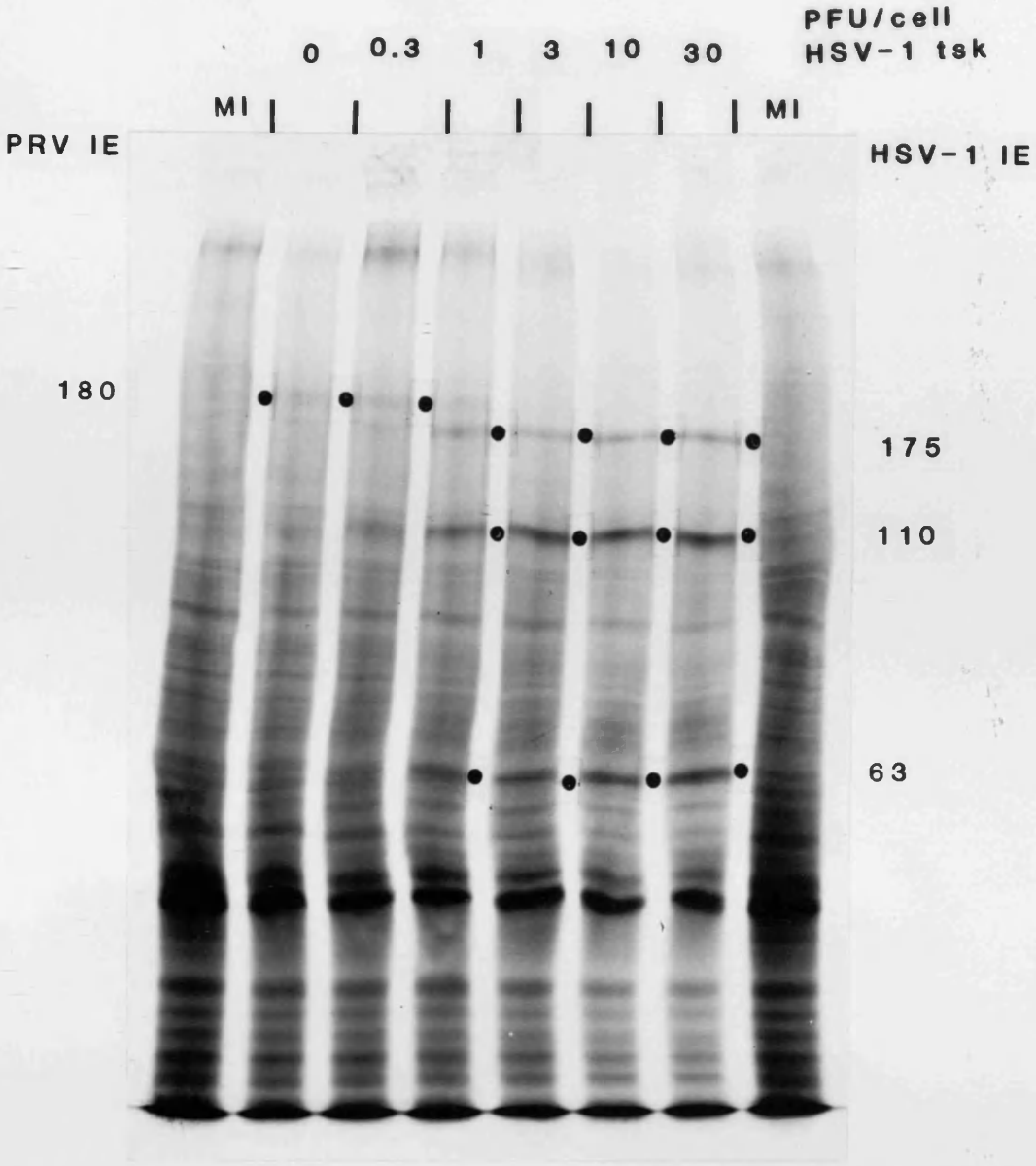

PFU/cell
HSV-1 tsk
0
0.3
1
3
10
30
MI
MI
PRV IE
HSV-1 IE
180
175
110
63

Figure 16. Co-infection with 30pfu PRV and various multiplicities of HSV-1 tsk per cell in the presence of 200ug/ml of cycloheximide.

Cells were labelled 4h after infection with ^{35}S methionine in the presence of of 2.5ug/ml actinomycin D and the absence of cycloheximide. Samples were electrophoresed on an 8% SDS polyacrylamide gel. The molecular weights of IE polypeptides are shown.

* Alternatively, there may be another HSV-1 virion component which supresses expression of the PRV IEP and cellular polypeptides.

permitted the translation of the IE gene products. Cells were radiolabelled by incubation with [^{35}S]-methioneine, harvested, and samples of the extracts were electrophoresed on SDS polyacrylamide gels. Figure 16 shows the polypeptide profiles obtained when cells were infected with 30pfu/cell of PRV plus various multiplicities of HSV-1. The PRV IEP was less efficiently expressed than the HSV IE polypeptides when equivalent multiplicities of infection were used. It was observed that HSV-1 infection did not induce synthesis of the PRV IE polypeptide but rather, the amount of this polypeptide produced was reduced when coinfection was carried out with greater than 1pfu/cell. These results demonstrate that the presence of the HSV-1 TIF does not stimulate expression mediated by the PRV IE gene regulatory sequences when they are present in total virus rather than chimaeric plasmids. The reason for this apparent contradiction has not been investigated further. However, it is possible that the PRV regulatory sequences are not accessible to Vmw65 when they are present within the virus particle, or that the HSV-1 IE control regions are outcompeting the control region of the PRV IE gene for Vmw65 or necessary cellular factors.*

7.6 Nucleotide sequence of the PRV IE gene upstream region

To investigate further the control of the PRV IE gene, the sequence of the region between the NcoI (+265) and HindIII (-526) sites was determined. This fragment was cloned into pUC9 to create pPRV4 (figure 14) and subfragments of the insert were cloned into M13 phage vectors which allowed the isolation of single stranded template. The nucleotide sequence of the inserts was determined by the dideoxy chain termination method and the position of overlapping fragments was verified by computer analysis. Figure 17 shows a listing of the gel readings, figure 18 shows the database obtained and a sample autoradiograph of a sequence ladder is depicted in figure 19. The final sequence is presented in figure 20.

Within the PRV IE upstream region a number of

CONTIG LINES

		a		b	c
	199	791	0	19	12

GEL LINES

d	e	f	g	h	i
B23.G12	19	1	108	0	17
B31.G14	17	1	-109	19	9
H1.G5	9	3	107	17	21
H4.G4	21	8	-103	9	22
B8.GA2	22	63	-102	21	29
C80.G21	29	101	68	22	33
H10.G4A	33	108	-71	29	20
A23.G16	20	139	-87	33	7
h10.g4	7	146	-38	20	16
H39.G15	16	146	-124	7	3
B8.G2	3	146	-187	16	15
H31.G13	15	169	-163	3	8
J51.G7	8	195	-140	15	30
C98.G21	30	215	181	8	6
J5.G4	6	249	-103	30	14
A22.G11	14	300	150	6	2
B2.G2	2	329	-151	14	28
C24.G16	28	332	148	2	27
C22.G16	27	333	-146	28	24
C56.G19	24	357	-215	27	26
C55.G18	26	444	-150	24	31
C55.G19	31	445	-159	26	23
H37.G15	23	479	65	31	4
A7.G3	4	502	-107	23	18
J4.G5	18	534	-42	4	1
B16.G2	1	582	-210	18	32
C90.G21	32	610	181	1	5
A2.G3	5	628	-162	32	11
T1.G15	11	663	-129	5	13
T3.G7	13	680	-112	11	12
A21.G11	12	681	-111	13	0

Figure 17. Contig and gel lines.

Contig Lines	a	length of contig (bp)
	b	database number of gel at extreme left of contig
	c	database number of gel at extreme right of contig

The other numbers have no particular significance.

Gel Lines	d	experimental name of clone
	e	gel database number
	f	position of left base of gel reading in the contig
	g	length of gel reading (negative value indicates that the reading is the reverse orientation)
	h	number of gel overlapping on the right
	i	number of gel overlapping on the left

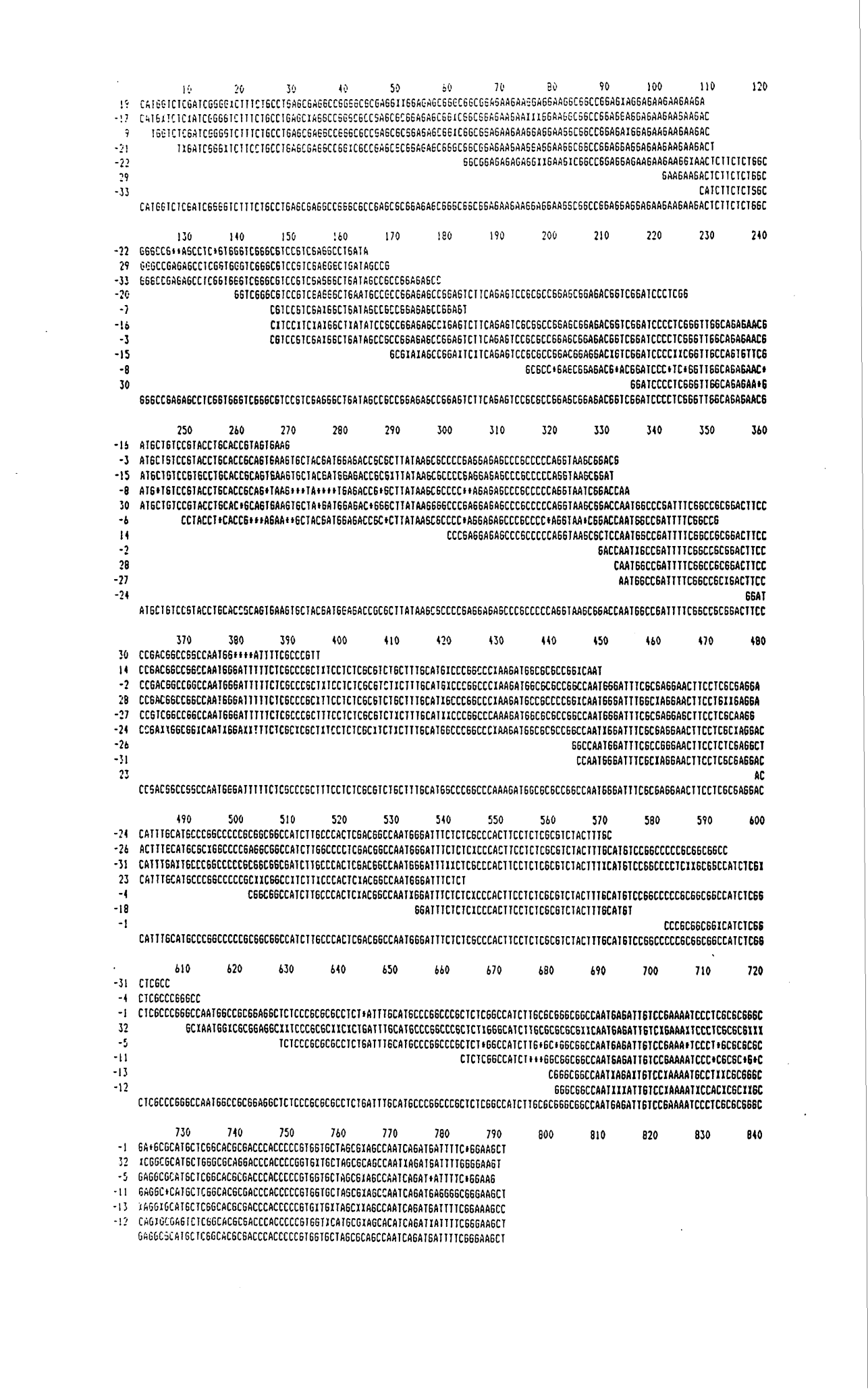


Figure 18. Database.

All gel readings entered in the database are shown. The figures at the left hand side are the gel numbers as listed in figure 17 column e. The consensus sequence is presented below the gel readings.
*, "padding character" inserted by the program; X, "padding character" inserted by operator.

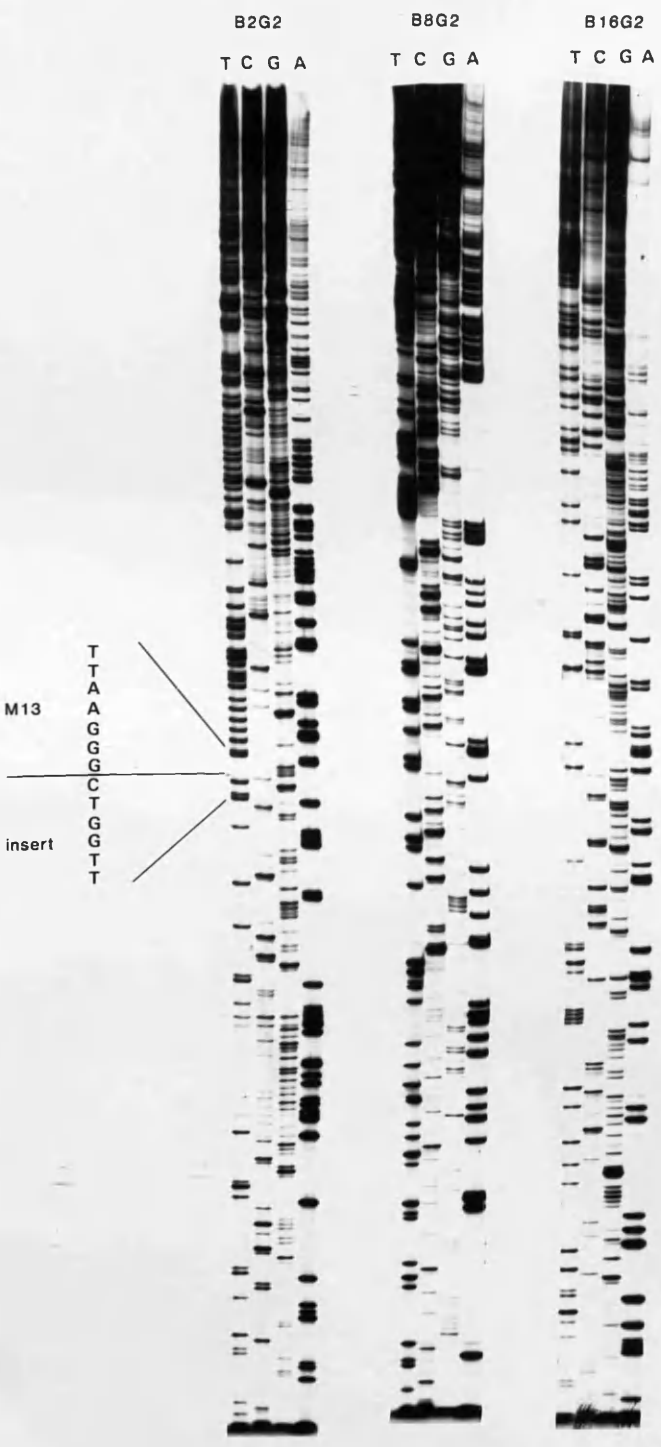

B2G2
T C G A
B8G2
T C G A
B16G2
T C G A
M13
T T A A G G G C T G G T T
insert

Figure 19. Example of sequence autoradiograph.

Clones are numbered as indicated in the gel lines (figure 17). The junction between M13 vector sequence and insert sequence is indicated for clone B2G2. Samples were electrophoresed on a gradient denaturing gel (section 5.7.4).

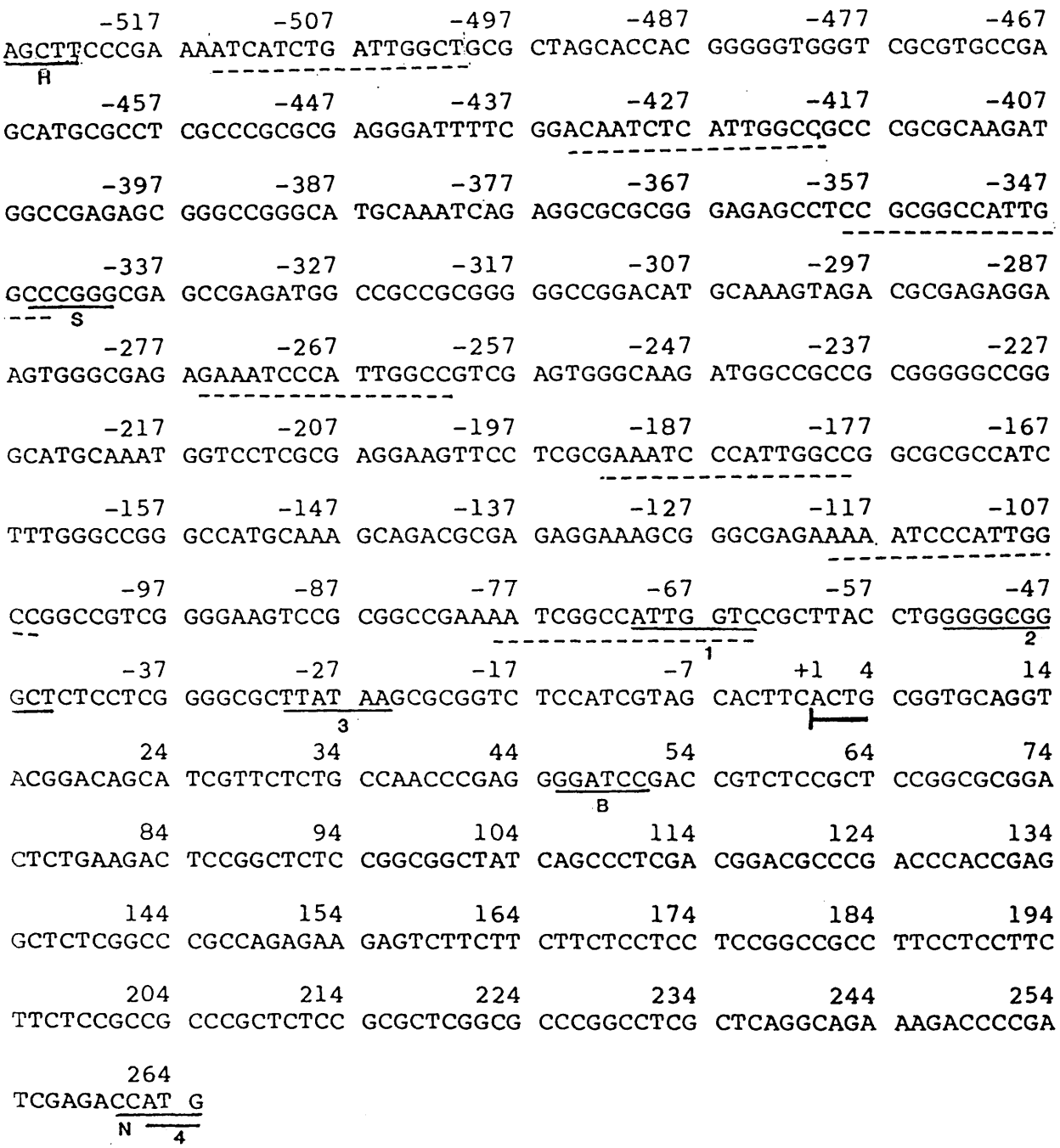

-517 -507 -497 -487 -477 -467
AGCTTCCCGA AAATCATCTG ATTGGCTGCG CTAGCACCAC GGGGGTGGGT CGCGTGCCGA
R
-457 -447 -437 -427 -417 -407
GCATGCGCCT CGCCCGCGCG AGGGATTTTC GGACAATCTC ATTGGCCGCC CGCGCAAGAT
-397 -387 -377 -367 -357 -347
GGCCGAGAGC GGGCCGGGCA TGCAAATCAG AGGCGCGCGG GAGAGCCTCC GCGGCCATTG
-337 -327 -317 -307 -297 -287
GCCCGGGCGA GCCGAGATGG CCGCCGCGGG GGCCGGACAT GCAAAGTAGA CGCGAGAGGA
S
-277 -267 -257 -247 -237 -227
AGTGGGCGAG AGAAATCCCA TTGGCCGTCG AGTGGGCAAG ATGGCCGCCG CGGGGGCCGG
-217 -207 -197 -187 -177 -167
GCATGCAAAT GGTCCTCGCG AGGAAGTTCC TCGCGAAATC CCATTGGCCG GCGCGCCATC
-157 -147 -137 -127 -117 -107
TTTGGGCCGG GCCATGCAAA GCAGACGCGA GAGGAAAGCG GGCGAGAAAA ATCCCATTGG
-97 -87 -77 -67 -57 -47
CCGGCCGTCG GGGAAGTCCG CGGCCGAAAA TCGGCCATTG GTCCGCTTAC CTGGGGGCGG
1 2
-37 -27 -17 -7 +1 4 14
GCTCTCCTCG GGGCGCTTAT AAGCGCGGTC TCCATCGTAG CACTTCACTG CGGTGCAGGT
3
24 34 44 54 64 74
ACGGACAGCA TCGTTCTCTG CCAACCCGAG GGGATCCGAC CGTCTCCGCT CCGGCGCGGA
B
84 94 104 114 124 134
CTCTGAAGAC TCCGGCTCTC CGGCGGCTAT CAGCCCTCGA CGGACGCCCG ACCCACCGAG
144 154 164 174 184 194
GCTCTCGGCC CGCCAGAGAA GAGTCTTCTT CTTCTCCTCC TCCGGCCGCC TTCCTCCTTC
204 214 224 234 244 254
TTCTCCGCCG CCCGCTCTCC GCGCTCGGCG CCCGGCCTCG CTCAGGCAGA AAGACCCCGA
264
TCGAGACCAT G
N 4

Figure 20. Nucleotide sequence of the PRV IE gene from -526 to +265.

Position +1 indicates the 5' end of the IE mRNA as defined by S1 nuclease mapping. Specific features of the sequence are as follows: solid lines, H, HindIII; S, SmaI; B, BamHI; N, NcoI; 1, inverted "CCAAT" box; 2, expected Spl binding site; 3, "TATA" homology; 4, potential ATG initiation codon; dashed lines, 15bp repeated elements, as shown in the inverted orientation in table12.

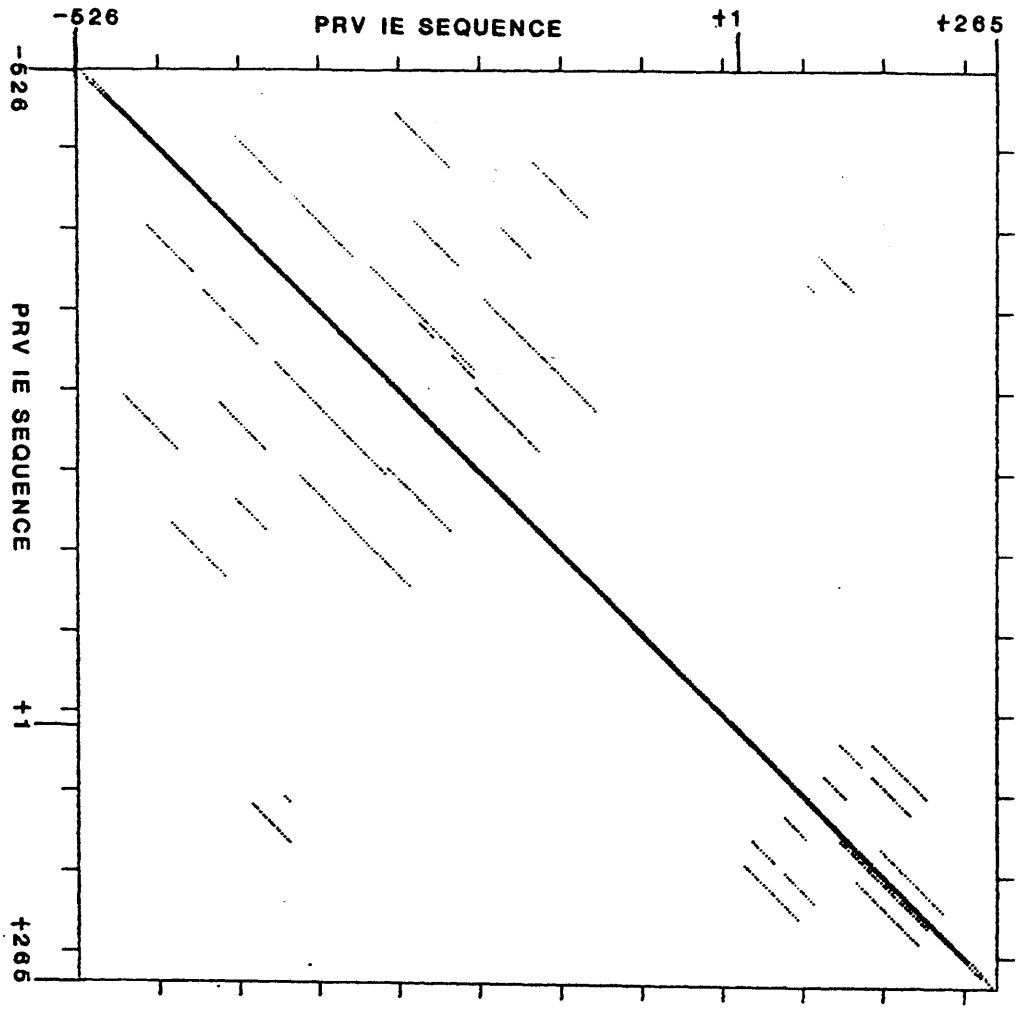

-526
PRV IE SEQUENCE
+1
+265
-526
PRV IE SEQUENCE
+1
+265

Figure 21. Homology matrix plot of PRV IE upstream sequences.

Self comparison of PRV IE sequences from -526 to +265 as depicted in figure 20. 1 division = 70 nucleotides; range = 50 nucleotides; minimum value plotted = 45%; compression = 7 times.

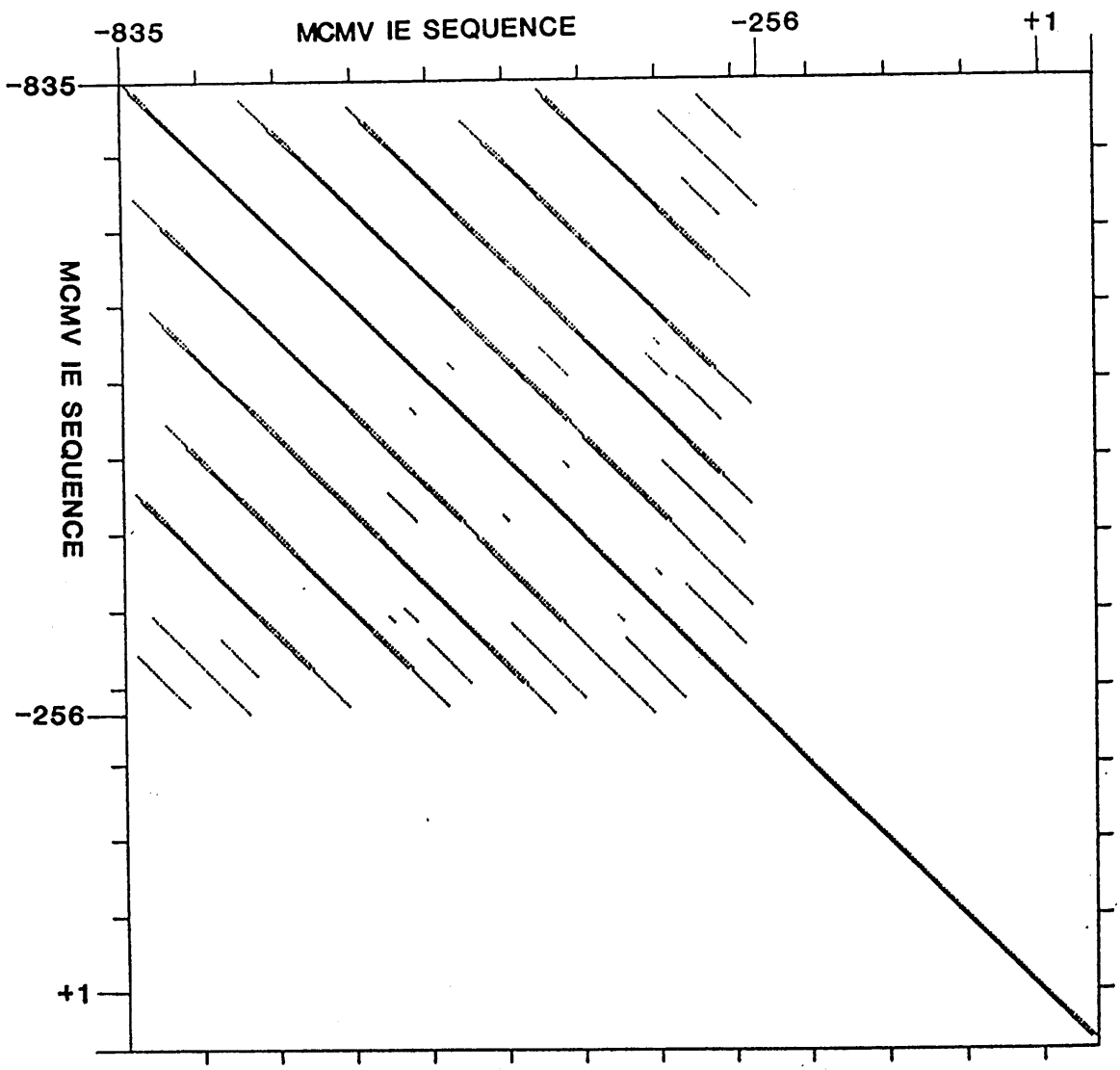

-835
MCMV IE SEQUENCE
-256
+1
-835
MCMV IE SEQUENCE
-256
+1

Figure 22. Homology matrix plot of MCMV IE upstream sequences.

Self comparison of the MCMV IE sequences from -835 to +50. 1 division 70 nucleotides; minimum value plotted = 45%; compression = 7 times.

sequence elements which have been implicated in eukaryotic transcriptional control can be detected. The sequence TTATAA is located 25bp upstream from the 5' end of the IE transcript and probably represents the 'TATA' homology found in an equivalent position in many eukaryotic and viral genes (Corden et al., 1980; section 3.1). The sequence between position -53 and -44, GGGGCGGGCT, has a high degree of homology with the consensus sequence binding sites for the transcription factor Spl (Kadonaga et al., 1986; section 3.2). Upstream regions of HSV IE genes are rich in Spl binding sites, five being present upstream of HSV-1 IE gene 3 (Jones and Tjian, 1985). The sequence downstream of position -70 in the PRV IE gene, when inverted (GACCAAT), is a perfect match for the 'CCAAT' box (RRCCAAT) which is found approximately 80bp upstream of the mRNA start site in a number of genes transcribed by RNA polymerase II (Efstratiadis et al., 1980; Benoist and Chambon, 1981; section 3.2).

A homology matrix plot was constructed where both the abscissa and ordinate represent the PRV IE upstream sequence (figure 21). This plot reveals the upstream region to be composed of a series of degenerate repeats of approximately 80bp in length, situated between positions -526 and -106. The most prominent feature of these reiterations is the 15bp element RAAATCCCATTGGCC, of which there are three exact copies at positions -275, -192 and -119. A copy with 2 mismatches occurs at position -434, and copies with lower homology are present at -514, -350 and -78. It was observed that the inverted orientation of this sequence overlaps with an 18bp repeated element which occurs in the upstream region of the HCMV and MCMV major IE genes, as shown in Table 12. In addition, the element at -434, in inverted orientation, also showed homology with the HSV TAATGARATTC motif.

7.7 Discussion

The 5' end of the PRV IE mRNA was accurately mapped to 50bp upstream of the BamHI site between fragments e and i. This result improves on that obtained previously by Ihara et al. (1983) who estimated, by Southern blot analysis, that

the mRNA terminus was located in the 750bp XhoI/KpnI fragment of KpnI h.

The data obtained, showing UV inactivated PRV to be ineffective in the stimulation of HSV IE genes, agrees with those obtained previously using infectious PRV (Batterson and Roizman, 1983). They also show that there is no specificity of PRV polypeptides towards homologous IE gene regulatory sequences since, surprisingly, it was found that PRV IE regions contained within pTKPRV1, pTKPRV2 and pTKPRV3 were responsive to the HSV TIF, whether delivered in the form of UV inactivated virus or transfected plasmid. It was therefore concluded that the basis for the difference between the properties of PRV and HSV derived from the absence or inactivity of a polypeptide equivalent to Vmw65.

A comparison of nucleotide sequence data revealed that VZV possessed a homologue of Vmw65 (Dalrymple et al, 1985). In cotransfection assays a plasmid bearing the encoding gene was unable to induce HSV IE expression (Dalrymple, 1986). However, further control experiments need to be carried out to ensure the gene is adequately expressed in BHK cells. It is therefore possible that PRV also contains a polypeptide homologous to Vmw65 which performs a structural role but is inactive in trans-activating function. However, envelope/tegument preparations of PRV show no unglycosylated species present in equivalent amounts to Vmw65 of HSV-1 (Hampl et al, 1984; Johnson et al, 1984). To determine whether any putative PRV homologue of Vmw65, which is present only in very low amounts in the virus particle, can trans-activate IE genes it will be necessary to identify and map the gene involved by the cotransfection approach used for HSV-1, as described in section 6.

PRV grows rapidly and efficiently in a wide variety of tissue culture lines and host organisms (Ben-Porat and Kaplan, 1985), showing that the presence of a TIF is not an absolute requirement for herpesvirus replication. One explanation of this difference between two otherwise biologically similar viruses may be that a TIF is required for the synchronous induction of the five HSV IE genes,

while PRV, which possesses only one major IE gene may not require such a mechanism.

The situation with regard to other herpesviruses has not yet been fully resolved. Bovine mamillitis virus and HCMV were ineffective in the induction of HSV IE genes (Batterson and Roizman, 1983; Stinski and Roehr, 1985) but the HCMV major IE gene was inducible by superinfection with HCMV in the presence of cycloheximide, or when the virus is UV inactivated, suggesting that HCMV, like HSV possesses a TIF (Spaete and Mocarski, 1985; Stinski and Roehr, 1985). The gene encoding this polypeptide has not yet been identified. Spaete and Mocarski (1985) also report that superinfection with HSV-1 stimulates expression of the HCMV major IE gene, however, this claim is not supported by other workers (Stinski and Roehr, 1985).

The nucleotide sequence of the PRV region upstream to -526 is notable for the presence of an imperfect repeated element between 75 to 85 bp in length. This general pattern is unlike HSV but is strikingly similar to the enhancers of the HCMV and MCMV, which are also located upstream from the major IE gene (Boshart et al, 1985; Dorsch-Hasler et al, 1985; Stinski and Roehr, 1985), as can be observed from a comparison of homology matrix plots of the 5' regions of PRV and MCMV IE genes (figures 21 and 22). Within the PRV sequence a 15bp element is found to be highly conserved. This repeated element of PRV is homologous to a conserved 18bp element present in the cytomegaloviruses (table 12). In MCMV, this conserved element is repeated at intervals of 92 or 93bp, but in HCMV no regular pattern can be discerned. It is thought, however, that the 18bp element is an important functional domain of the HCMV enhancer, and that the repetition of conserved motifs is involved in attaining enhancer function (Weber et al., 1983; Serfling et al., 1985; Stinski and Roehr, 1985). The family of repeated elements present in PRV, HCMV and MCMV also show homology to the HSV consensus TAATGARATTC, which is of vital importance in the response to TIF. The PRV sequence has therefore provided a link to reveal unexpected similarities between

herpesviruses with such diverse biological properties that they are classified into different sub-families (HSV and PRV are alphaherpesvirinae, HCMV and MCMV are betaherpesvirinae).

The PRV IE gene has a CCAAT box in the inverted orientation at -78. This promoter element is found in many eukaryotic genes approximately 80bp from the start of the RNA transcript (Efstratiadis et al., 1980; Benoist and Chambon, 1981). The upstream repeated PRV elements, along with other sequence motifs shown in table 12 also contain CCAAT homologies. An inverted CCAAT box upstream of the HSV-1 TK gene has been shown to be involved in transcriptional control and also to bind a cellular factor (Jones et al., 1985; Graves et al.,1986; McKnight and Tjian, 1986). The CCAAT homologies shown in table 12 may contribute to cis activation of their respective genes by binding cellular factors. The involvement of cellular transcription factors in the expression of the HCMV major IE gene was demonstrated by the observation that synthesis of the IE polypeptide occurred in human carcinoma cells only after they were induced to differentiate (LaFemina and Hayward, 1986). This type of regulation is similar to that found for the polyoma virus enhancer, as discussed in section 3.3.3 and exemplifies the interaction of cell specific factors with the HCMV regulatory sequences.

The experiments described here show that PRV sequences between +48 and -342 respond to the HSV TIF. A recent detailed analysis has identified two DNA elements involved in the response of HSV-1 IE gene 3 to the TIF (Bzik and Preston, 1986). One of these was TAATGARATTC, which was sufficient for trans-activation if present in close agreement with the consensus, and a second was a GA-rich element. The GA-rich element was able to activate a TAATGARATTC-like motif which matched the consensus poorly and was itself nonfunctional in isolation. Based on this information, two parts of the PRV IE gene upstream region (-217 to -261 and -373 to -416) have a TAATGARATTC-like sequence plus a GA-rich flanking region, and it would be expected that these mediate the response to TIF. A detailed deletion analysis of the PRV upstream region will be

necessary to determine precisely the elements which respond to the HSV TIF.

Table 12. Comparison of IE upstream repeated elements from MCMV, HCMV, HSV and PRV.

Orientation			Position
+	MCMV	GYCAATAGGGACTTTCCAT	(consensus)
+	HCMV	C/ACTAACGGGACTTTCCAA	(consensus)
+	HSV	GNTAATGARATTC	(consensus)
-	PRV	GGCCAATGGGATTTY	(consensus)
-	PRV	AGCCAATCAGATGAT	-500 to -514
-	PRV	GGCCAATGAGATTGT	-420 to -434
-	PRV	GGCCAATGGCCGCGG	-344 to -358
-	PRV	GGCCAATGGGATTTC	-261 to -275
-	PRV	GGCCAATGGGATTTC	-178 to -192
-	PRV	GGCCAATGGGATTTT	-105 to -119
-	PRV	GACCAATGGCCGATT	- 64 to - 78
+	"CCAAT" box	RRCCAAT	

Sources of consensus elements are as described in the text. PRV sequences are shown in figure 5 but represented in the inverted orientation. The orientation (+ or -) is described with reference to the 5' to 3' direction of the relevant mRNA.

8 PURIFICATION AND CHARACTERISATION OF VMW65

8.1 Introduction

This section describes preliminary attempts to purify and characterise Vmw65 in order to elucidate the way in which it effects transcriptional regulation.

The results described in section 6.7 demonstrate that Vmw65 is likely to be a structural component, as it is immunoprecipitated by the monoclonal antibody MA1044 which reacts with a polypeptide from purified virions (J.W. Palfreyman, personal communication). Analysis of polypeptide profiles shows Vmw65 to be a major constituent of HSV-1 virions and suggests that each virion contains between 850 and 1,200 molecules of this species (Spear and Roizman, 1972; Heine et al., 1974). Experiments were therefore performed in an attempt to isolate Vmw65 from HSV-1 virions. A 65,000 mw polypeptide, which possessed strong DNA binding properties, was also purified from infected cell extracts by Dr H.S. Marsden.

In the following sections the two methods of extraction of a 65,000 mw polypeptide and the properties of the fractions obtained will be discussed.

8.2 Extraction of a 65,000 mw polypeptide from HSV-1 virions by NP40 treatment.

Previous work had shown that HSV-1 virions were disrupted by incubation with non-ionic detergent such as NP40 (Olshevsky and Becker, 1970; Dreesman et al., 1972; Spear and Roizman; 1972 Johnson et al., 1984). Based on this information, preparations of surface and tegument polypeptides were obtained using the following procedure: Samples of ^{35}S labelled virions were incubated with 0.03% NP40, 50mM NaCl, 10mM Tris (pH7.5), 1mM EDTA at 4°C for 3h and subjected to high speed centrifugation to pellet the capsids. The polypeptides in the supernatant and intact virion preparations are shown in figure 23. A 65,000 mw species was a major constituent of the total virions and also of the NP40 extract and the only other major constituents of the extract were the glycoproteins gB

and gD.

8.3 Purification of a 65,000 mw DNA binding protein from HSV-1 infected cells

The second method used to obtain an extract containing HSV-1 specific 65,000 mw polypeptide utilised infected cells and was prepared by Dr. H. S. Marsden (Marsden et al., 1987). A high salt (2M) extract was prepared from HSV-1 infected cells, dialysed to remove excess salt and loaded on a calf thymus DNA cellulose column. The column was eluted with a 0.05 to 0.9M NaCl gradient and finally with a 2M NaCl step. A 65,000 mw DNA binding protein ($65K_{DBP}$) was eluted across the range of salt concentrations and was found to be substantially pure in the 2M step.

8.4 Identification of Vmw65 in virus preparations

Prior to the investigation of the properties of Vmw65 it was necessary to determine whether the 65,000 mw polypeptide in either or both of the virus preparations described in sections 8.2 and 8.3 was the TIF, Vmw65. This was achieved by immunoprecipitation of the NP40 supernatant and the DNA binding extract with the monoclonal antibody LP1 (McLean et al., 1982). This antibody was directed against an HSV-2 65,000 mw polypeptide but like MA1044, will spcifically precipitate HSV-1 TIF (section 6.7.) (Marsden et al., 1987). LP1 precipitated the 65,000 mw polypeptide from the NP40 virion extract (figure 24, track 3), showing this species to be Vmw65, the TIF. The $65K_{DBP}$ was not precipitated. Both of the samples containing the 65,000 mw polypeptide were also reacted with an antiserum (13810) which was directed against the DNA binding extract (Marsden et al., 1987). The only 65,000 mw polypeptide to react with the antiserum was that from the DNA binding extract (track 8). These results demonstrate that Vmw65 and $65K_{DBP}$ are antigenically distinct. The presence of gB in the 13810 precipitate as explained by the specificity of this antiserum for gB (Marsden et al., 1987), while gB in the LP1 precipitates may be due to the occurrence of gB and Vmw65 in

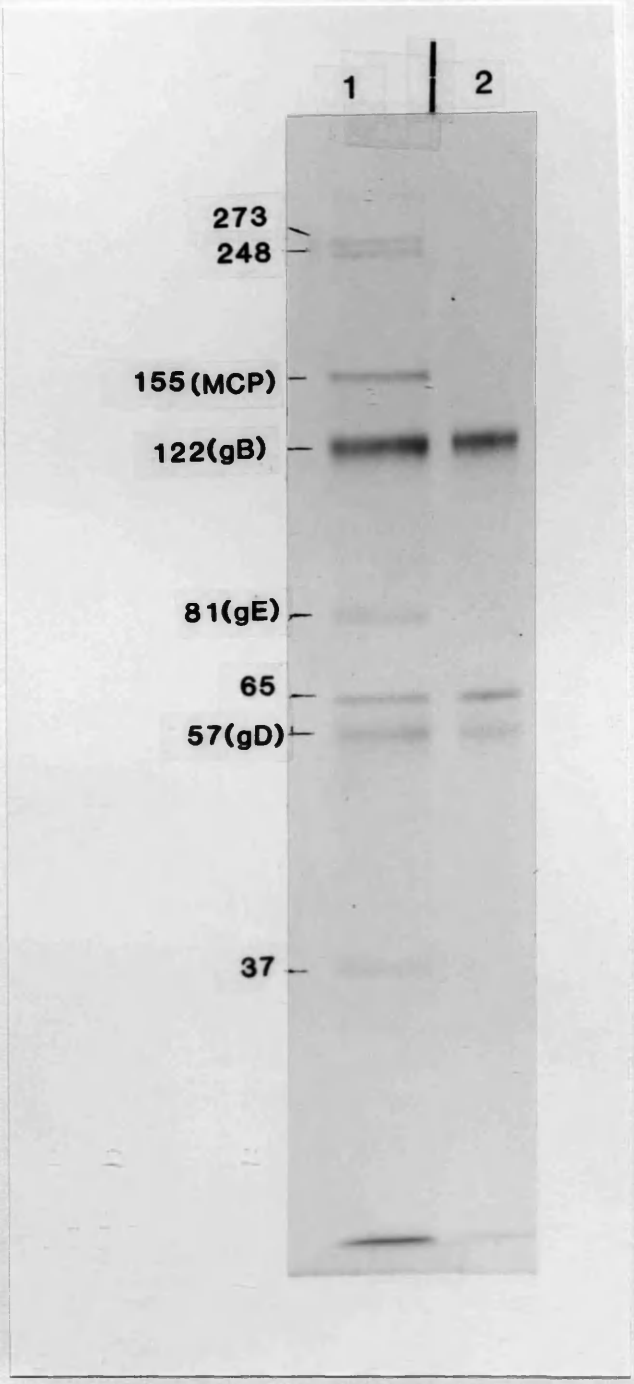

1
2
273
248
155(MCP)
122(gB)
81(gE)
65
57(gD)
37

Figure 23. NP40 extract of HSV-1 virions.

Total ^{35}S methionine labelled virions (track 1), supernatant of high speed centrifugation following incubation of virions with 0.03% NP40 (track 2). Samples were electrophoresed on a 10% SDS polyacrylamide gel. The molecular weights (X 10^3) of the polypeptides are indicated.

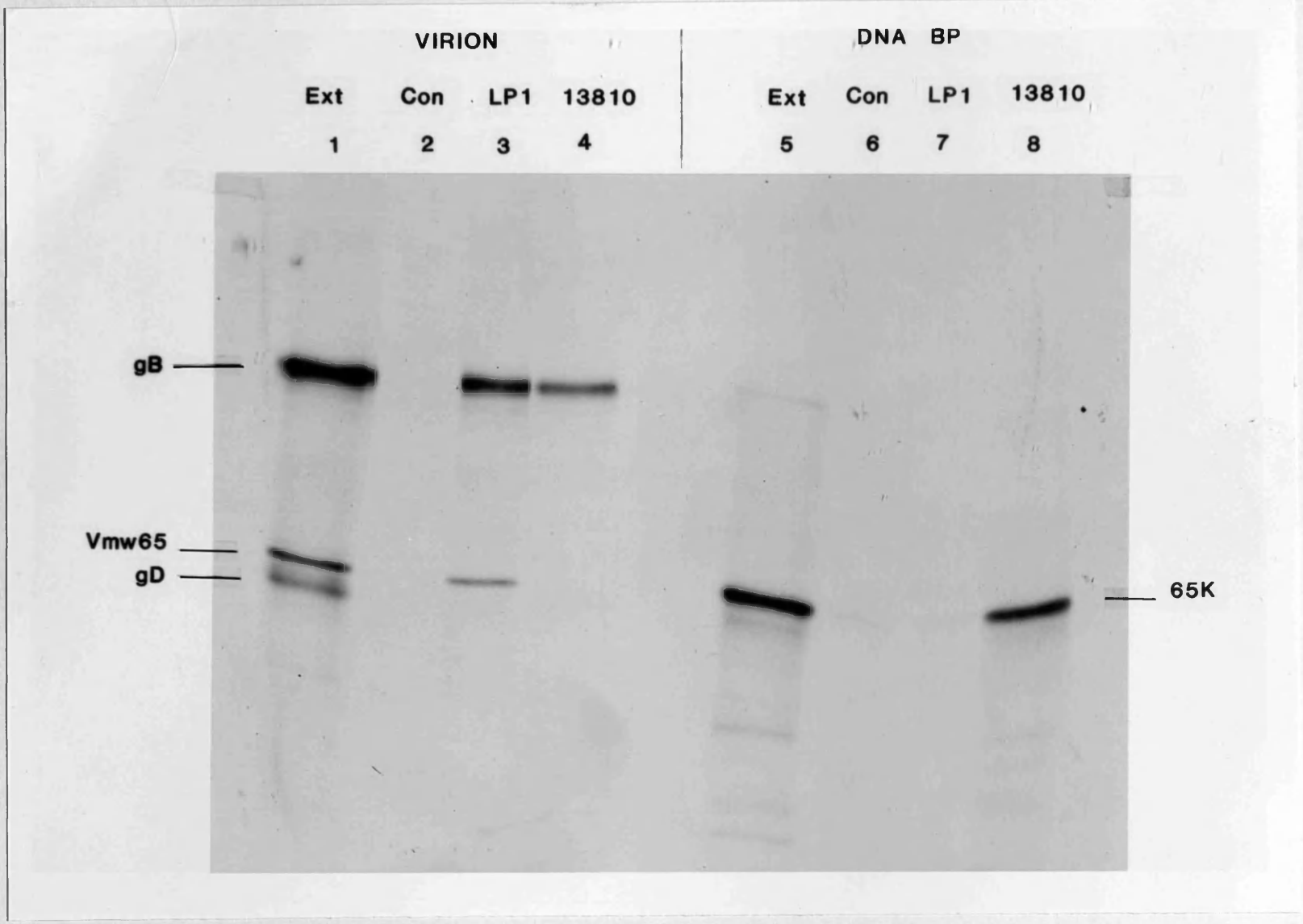

VIRION
DNA BP
Ext
Con
LP1
13810
Ext
Con
LP1
13810
1
2
3
4
5
6
7
8
gB
Vmw65
gD
65K

Figure 24. Immunoprecipitation of extracts containing HSV-1 induced polypeptides of mw 65,000.

NP40 virion extract (tracks 1-4), DNA binding extract from HSV-1 infected cells (tracks 5-8), total sample (tracks 1 and 5), precipitated with control ascites fluid (tracks 2 and 6), precipitated with monoclonal antibody LP1 (tracks 3 and 7), precipitated with 13810 antisera (tracks 4 and 8).

a complexed form, or because the two peptides share an antigenic site.

8.5 Attempted purification of Vmw65 by a calf thymus DNA cellulose column

The results described in section 8.4 show that the 65,000 mw HSV-1 polypeptide with strong DNA binding properties is not Vmw65. It was not known, however if Vmw65 associated with DNA. The interaction of the NP40 extract with DNA cellulose was therefore examined, for two reasons. First, it was hoped that this procedure could be used as a purification step, and second, the interaction of Vmw65 with cellular DNA would imply that its trans-activating properties were conferred by direct binding to regulatory sequence elements.

A sample of NP40 extract was passed through a column containing double stranded calf thymus DNA in a cellulose matrix. After washing with low salt buffer the column was eluted with various salt concentrations. Figure 25 shows 0.15M and 0.6M NaCl eluates from the column together with the input and flowthrough samples. No detectable polypeptides were observed in either eluate. A separate experiment was carried out in which the column was eluted with stepwise increases in salt concentration between 0.1M and 1M NaCl. Again, no polypeptides were eluted in any of the fractions and no significant levels of radioactivity were found in any of the eluted fractions. As a control, an extract of BHK cells obtained under high salt conditions was added to an identical DNA cellulose column. The pattern of elution of cellular polypeptides with increasing salt concentration was similar to that obtained previously (Hay, 1979), showing the column to be efficient in the retention of DNA binding species (results not shown). The Vmw65 present in the NP40 extract of HSV-1 virions did not detectably bind to calf thymus DNA under the conditions used. It is possible, however, that the extraction procedure used inactivated the Vmw65 or that its interaction with gB prevented association with DNA.

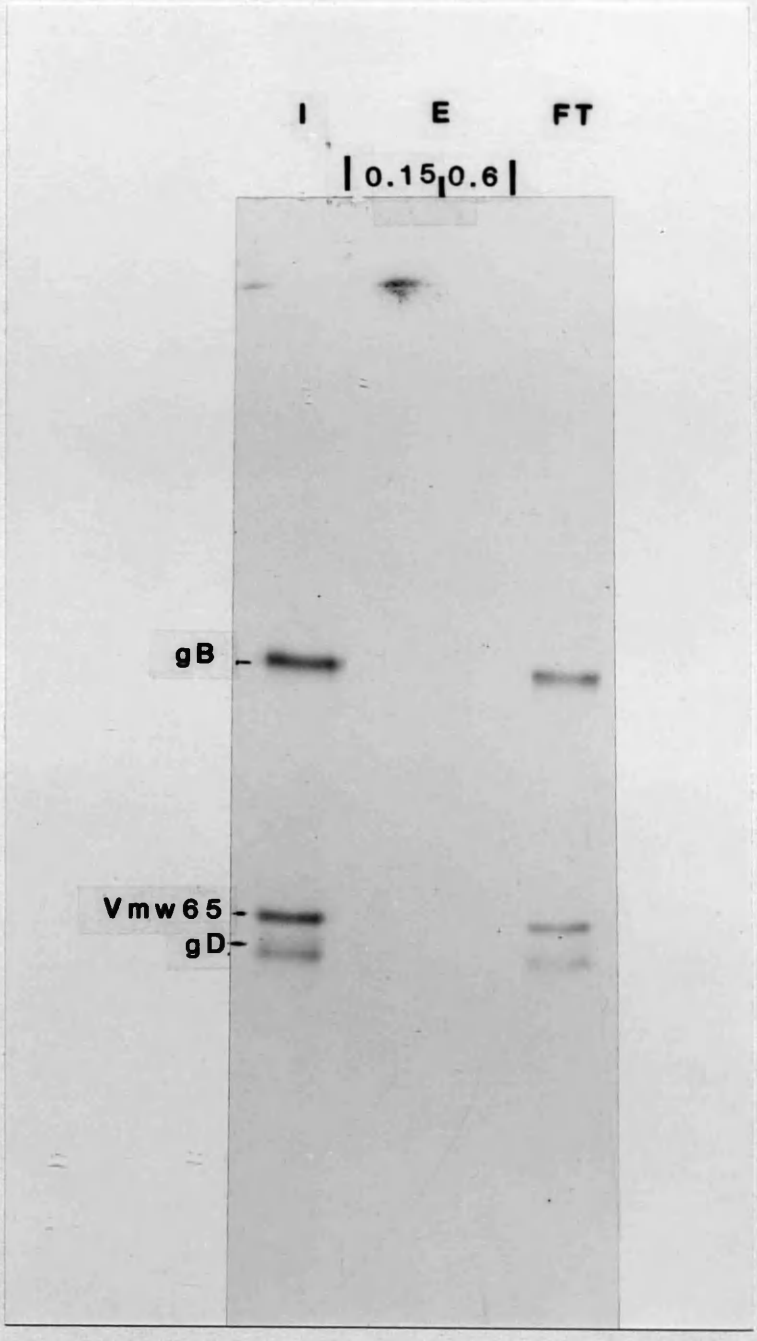

I
E
FT
0.15
0.6
gB
Vmw65
gD

Figure 25. Addition of NP40 extracts to calf thymus DNA cellulose column.

I, input; E, eluates (0.15 and 0.6M NaCl); FT, flowthrough.

8.6 Discussion

The experiments performed showed that a 65,000 mw polypeptide may be partially purified from HSV-1 virions by treatment with 0.03% NP40. Immunoprecipitation studies established that this polypeptide was the TIF, Vmw65. A 65,000 mw HSV-1 induced DNA binding protein, present in infected extracts was found to be immunologically distinct from Vmw65. The fact that these polypeptides are different species has been verified by the comparison of tryptic peptide maps and 2-D gel electrophoresis (Marsden et al, 1987) and the gene encoding $65K_{DBP}$ has now been identified (figure 4),(D. Parris, personal communication).

Treatment of the virion extract with monoclonal antibody directed against Vmw65 also resulted in the precipitation of gB. It therefore seems probable that Vmw65 occurs in a complex with this glycoprotein in the NP40 extract. The association of Vmw65 with glycosylated species was also observed by Johnson et al. (1984) in acetyl glycoside extracts of HSV-1 virions. In addition, these workers found that protease treatment of intact virions resulted in the degradation of gB and gD but not Vmw65, indicating that the latter polypeptide is not exposed on the virion surface. Vmw65 is found in association with nucleocapsids following the solubilisation of the virion envelope (Gibson and Roizman, 1972; Lemaster and Roizman, 1980) and may be therefore be envisaged as a matrix polypeptide located between the capsid and envelope. Vmw65 may be associated with polypeptides of both structures.

The most obvious model to explain the trans-activation of HSV IE genes predicts specific binding of Vmw65 to IE upstream sequences. This type of interaction is found in other systems where a particular sequence element is required to mediate the response to a trans-acting factor. For example, the cellular factor, NF1, is found to markedly stimulate the replication of adenovirus DNA. This factor binds to sequences with the consensus TGGA/CNNNNNGCCAA (Borgmeyer et al., 1984; Nowock et al., 1985), a copy of which is located at the adenovirus replication origin (Rawlings et al., 1984; Leegwater et al.,

1985). Transcriptional activation is mediated by interaction of cellular factors, including Spl, CTF, HSF and TATA binding factor with specific cis-acting sequences located 5' to the mRNA initiation site, as discussed previously (sections 3.1, 3.2 and 3.4.2).

Sequence-specific DNA binding factors are found to bind total cellular DNA, but will interact with a greater affinity to the responding sequence (Payvar et al., 1981; Mulvihill et al, 1982; Rawlings et al., 1984). No evidence was found, however, for the interaction of Vmw65 in virion extracts with calf thymus DNA. Vmw 65 present in infected cell extracts was also tested for its affinity for calf thymus DNA cellulose (Marsden et al, 1987). Immunoprecipitation with monoclonal antibody LP1, showed Vmw65 to be present in the input fraction but not in 0.15M or 0.6M eluates, or in the 2M eluate (this study, figure 24). The fact that the same result was obtained using extracts obtained by two different methods argues that the lack of DNA binding properties of Vmw65 is not due to inactivation during the extraction procedure.

These studies indicate that Vmw65 is unlikely to mediate transcriptional activation via direct sequence interaction. The possibility remained, however, that this protein only bound to specific HSV activator sequences. This hypothesis has been ruled out by recent studies which show that Vmw65 in virion extract was unable to bind a 77bp DNA fragment from the upstream region of IE gene 4/5, which contains a functional TAATGARATTC sequence (Preston et al., submitted). A second possibility is that Vmw65 requires to bind a cellular factor prior to its interaction with DNA sequence elements. This is considered to be the mechanism by which the viral trans-activators Vmw175 and E1A proteins operate (sections 2.3.1 and 3.5.1). Steroid hormones, such as glucocorticoid and progesterone, interact with specific target sequences via the prior formation of a complex with receptor proteins (Payvar et al., 1982; Mulvihill et al., 1982; section 3.4.2). Studies relating to the interaction of Vmw65 with a cellular factor are discussed in section 9.

9 FINAL DISCUSSION

The most important aspect of the work carried out was the identification of Vmw65 as the virion polypeptide responsible for the trans-induction of IE genes. This finding contributes to our understanding of the control of HSV gene expression and, because both trans-acting protein and cis-acting DNA sequences have been identified, the regulation of IE genes provides a useful model for the study of eukaryotic gene control.

In order to elucidate the mechanism by which Vmw65 stimulates IE gene transcription two courses of action are being followed at present. The first is the attempted creation of a viral mutant, defective in trans-inducing potential, and the second, a study of the in vitro binding of viral and cellular polypeptides to IE regulatory sequences.

Production of a mutant, with a trans-inducing defect is hindered by the fact that Vmw65 is a bifunctional polypeptide. The mutation within ts2203 is believed to lie within the coding sequences of the HSV-2 homologue of Vmw65 as the defect may be rescued by pMC1, and secondly, because the 65,000 mw polypeptide exhibits altered electrophoretic mobility compared to wild type. A cloned copy of the ts2203 gene encoding Vmw65 is, however, effective in the trans-induction of IE genes (V.G. Preston, M.A. Dalrymple, personal communication). The mutant, ts2203, is defective in the encapsidation of virus particles at NPT (F.H. Ramsay, personal communication), showing Vmw65 to be essential for the integrity of the virion, in addition to fulfilling a regulatory function. In order to investigate which regions of the polypeptide are involved in structure and trans-induction, a series of pMC1 linker insertion mutations have been constructed which do not alter the reading frame of the encoded polypeptide (C.I. Ace, personal communication). The results obtained revealed that distinct domains of Vmw65 are important for structure and for gene regulation, but certain regions are required for both functions. A region flanking the unique PvuII site of pMC1

was found to be sensitive to mutation and to be highly conserved between HSV and VZV (Dalrymple et al., 1985). One mutant which contained an insertion at amino acid 379 was able to rescue ts2203 but lacked trans-inducing function, indicating that the regulatory role of Vmw65 may not be essential for virus growth in tissue culture cells. However, further characterisation of this recombinant is necessary to ensure that it contains a linker insertion and is defective in trans-activation. Possible work for the future includes a more detailed investigation of the essential domains of Vmw65 by creating single base pair substitutions within the gene.

As previously discussed (section 8) Vmw65 is unable to bind directly to DNA or specifically to IE upstream sequences, indicating that a prior interaction with one or more host polypeptides occurs. This theory has been investigated in our laboratory by the use of in vitro binding studies (Preston et al., submitted). Nuclear extracts were prepared from HeLa cells, which were either mock infected or infected with HSV-1 under IE conditions, and incubated with radiolabelled fragment from the upstream region of IE gene 4/5. When infected cell extract was used a unique slowly migrating complex (IEC) was observed upon electrophoresis in a non-denaturing gel. Moreover, this complex also occurred when mock infected nuclear extract was mixed with NP40 extract of virus particles (as described in section 8.2). Vmw65 was found to be a component of the IEC and immunologically purified Vmw65 was capable of promoting complex formation, indicating that it was the only essential viral constituent. Formation of the IEC was specific to DNA fragments containing the TAATGARATTC sequence and DNAse I footprinting and methyl protection experiments showed this motif to be involved in protein binding. Other workers have found cellular factors to interact with the TAATGARATTC sequence but found that binding was not affected by using HSV infected (as opposed to mock infected) extracts (Kristie and Roizman, 1987; R Thompson, personal communication). It appears unlikely that these complexes are related to the IEC.

In the future we hope to identify the host components required for IEC formation and to investigate their role in cellular gene expression and metabolism.

There have been no previous studies at the molecular level to determine how the interactions of two or more polypeptides form a sequence specific binding complex. However, a comparison may be made with the model for action of bacterial regulatory proteins. Genetic and X-ray crystallography studies (McKay and Steitz, 1981; Gorges and Adhya, 1985) showed that cyclic AMP binding converted the E. coli cyclic AMP repressor from a non-specific binding protein to one which interacted only with the operon sequences of the promoter, thereby regulating transcription. Comparable examples in eukaryotic systems include those of the steroid hormones, glucocorticoid and progesterone, which effect a transcriptional increase via their interaction with a receptor binding protein (Mulvihill et al., 1982; Payvar et al., 1983) However, unlike the cyclic AMP repressor the glucocorticoid receptor is observed to bind specifically to the DNA sequence required for regulation in the absence of hormone (Willmann and Beato, 1986). Godowski et al. (1987) observed that receptor protein deletion mutants constitutively activated transcription, indicating that the hormone il icits a conformational derepression of the wild type receptor. At present it appears that regulation of HSV IE gene expression occurs by a different mechanism from the examples cited. Vmw65 has no DNA binding activity in the absence of host factors and preliminary evidence suggests that the cellular component of the IEC does not have a high affinity for DNA as it may be extracted from the nucleus at low salt concentrations (M.E.M. Campbell, C.M. Preston, unpublished data). It therefore appears that affinity for IE regulatory sequences is generated only after complex formation between viral and cellular constituents.

The way in which the interaction of a complex with a specific DNA sequence activates gene expression remains unknown. Possible mechanisms include those previously discussed (section 3.3.5) for the activation of enhancers by the binding of a trans-acting factor, for example, the

polypeptides associated with upstream sequences may provide an entry site for transcription factors or, by looping out intervening DNA, they may influence the formation or efficiency of an initiation complex at the mRNA start site.

RNA viruses possess oncogenes which share homology with genes encoding cellular regulatory proteins and are believed to be derived from these genes (Bishop and Varmus, 1982). Recent evidence has shown that embryonal carcinoma stem cells contain a transcription factor analagous to adenovirus EIA (La Thangue and Rigby, 1987) This factor is believed to be involved in the control of cellular gene expression during differentiation. Vmw65 may likewise be the counterpart of a cellular protein and may operate by interacting in an existing cellular pathway. Support for this theory is provided by the results of Kemp et al. (1986). These workers screened a panel of cellular cDNA clones, derived from HSV-1 infected cells with mRNA from infected and mock infected cells. It was found that, in accordance with previous data (La Thangue et al., 1984), the expression of a number of cellular genes is increased upon infection. One such gene was found to be strongly induced in cells infected in the presence of cycloheximide, suggesting that it was activated in an IE specific manner. Furthermore, the gene was not expressed during infection with PRV, which lacks an effective TIF (Campbell and Preston, 1987; section 7). These results suggest that the transcription of the cellular gene is stimulated, like HSV IE genes, by Vmw65. The mapping and sequencing of this gene will determine whether it possesses regulatory sequences homologous to the TAATGARATTC motif. It may be speculated that the cellular gene is normally regulated by a homologue of Vmw65.

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