An Investigation of the Effect of Hexamethylene Bisacetamide on Herpes Simplex Virus Gene Expression.

by

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Summary

A number of functionally related chemicals have been identified which overcome the replication defect of *in*1814, an HSV-1 mutant with a 12bp insertion mutation that inactivates the virion transinducing factor, Vmw65. Hexamethylene bisacetamide (HMBA) and dimethylsulphoxide (DMSO) are potent inducers of terminal differentiation in cultured erythroleukaemic cells, and have also been shown to increase the speed and efficiency of reactivation of latent HSV-1 and HSV-2 after explantation of ganglia.

The work presented in this thesis demonstrates that the presence of 3-5mM HMBA in the cell culture medium, the optimum concentration for induction murine erythroleukaemic cell (MELC) differentiation, increases the titre of *in*1814 on human foetal lung (HFL) cells 500-fold, enabling the mutant to initiate infection almost as efficiently as wild type HSV-1. The titre of 1814R, the rescued "revertant" virus, was virtually unaffected by the presence of 5mM HMBA. Related compounds DMSO and hypoxanthine, which induce MELC differentiation, also significantly increased the titre of *in*1814. A number of known metabolites of HMBA did not increase the titre of *in*1814, either alone or in combination with various concentration of HMBA.

The effect of HMBA is not cell type specific, as it was reproduced in a number of cell lines including HFL, BHK and HeLa. Pre-treatment of cells with HMBA prior to infection had no effect on the subsequent titre of *in*1814, demonstrating that HMBA does not act by inducing stable cellular changes. HMBA exposure was shown to be required only transiently, immediately after adsorption, indicating that HMBA functions at the beginning of the virus life cycle.

RNA dot blot analysis revealed that the presence of HMBA resulted in an increase in immediate early (IE) RNA accumulation after infection of cells in the presence of cycloheximide, such that the RNA levels in *in*1814-infected cells approached values observed in wild type HSV-1-infected cells in the absence of HMBA. The observation that HMBA increases IE-RNA levels in the presence of cycloheximide, suggests that its target is a pre-existing cellular or viral component(s).

Southern blot analysis of nuclear DNA isolated from HFL cells infected with *in*1814 in the presence or absence of HMBA, demonstrated

that the transport of viral DNA to the cell nucleus was not affected by HMBA.

A range of plasmids with the chloroamphenicol acetyl transferase (CAT) gene under the control of various viral promoters were introduced in HFL cells by lipofection. In this transient expression system, no detectable promoter or sequence specificity was identified in the mode of action of HMBA since increased CAT activity was observed from IE, early and late HSV-1 promoters and from the human cytomegalovirus (HCMV) and SV40 enhancers, in the presence of 5mM HMBA.

The question of specificity was subsequently addressed using a range of mutant viruses which allowed direct investigation into the effect of HMBA on different promoters in the viral genome. These results demonstrated that HMBA does not substantially compensate for the absence of Vmw110 or adenovirus (Ad) E1a, nor does it act as or induce an active cellular homologue of Vmw175. Further analyses revealed that the HCMV enhancer and SV40 promoter/enhancer are responsive to activation by HMBA when integrated into the HSV genome.

Gel retardation analysis revealed that HMBA does not promote IEC formation with the TAATGARAT motif nor does it act as a "linker molecule", allowing mutant Vmw65, extracted from *in*1814 virions, to interact with Oct-1 and TAATGARAT. Therefore HMBA does not act as or induce a cellular homologue of Vmw65. In addition, HMBA-treatment of nuclear extracts did not promote complex formation at other protein binding motifs involved in regulation of transcription.

The HMBA effect on the titre of *in*1814 was not attributable to its demethylating properties as treatment with 5-azacytidine, an inhibitor of DNA methylation, did not increase the titre of *in*1814. Addition of 8μM 5-azacytidine actually decreased the titre of *in*1814, especially when present both prior to and after the addition of the virus, whereas little effect was seen on the titre of 1814R.

HMBA-mediated modulation of protein kinase C (PKC) activity did not appear to be responsible for the effect on the titre of *in*1814, as an inhibitor of HMBA-induced MELC differentiation, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), did not antagonize the HMBA effect on *in*1814 titre. In addition, exposure to leupeptin, which is known to inhibit the conversion of membrane bound inactive PKC to

the soluble activated form, also failed to antagonize the effect of HMBA on the titre of *in*1814.

The addition of 5mM HMBA to the cell culture medium increased the efficiency of calcium phosphate-mediated transfection approximately threefold, for both wild type HSV-1 and *in*1814 viral DNA.

In an *in vitro* transcription initiation system, extracts prepared from HMBA-treated HeLa cells did not show increased transcription from the following promoters recognised by RNA polymerase II:- HSV-1 IE-1 promoter; IE-4 promoter, in the presence or absence of the upstream sequences; HCMV IE promoter and the Ad major late promoter. Similarly, no increase in transcription of the the RNA polymerase III transcribed Ad VA genes using the HMBA-treated extract was observed compared to the activity of the untreated extract. The polar planar HMBA molecule does not function simply as a consequence of its overall negative charge as the inclusion of various concentrations of HMBA in the transcription reaction had an inhibitory effect.

A series of thymidine kinase (TK) transformed cell lines were constructed, with the HSV-1 TK gene under the control of different classes of HSV promoters. HMBA treatment did not have any dramatic effect on the level of expression from the HSV-1 TK, IE-3 or IE-4 promoters in the cellular genome. Therefore, the results obtained indicate that HMBA can have a different effect on the same promoter sequence depending on the structural environment of that promoter.

The results suggest that HMBA- and DMSO-mediated enhancement of reactivation from latency is due to a direct increase in IE RNA production, rather than demethylation. Determination of the molecular basis for the activity of HMBA on *in*1814 infection may reveal the primary events by which the compound affects the poorly understood process of reactivation. In addition, these studies demonstrate a primary effect of HMBA on gene regulation which may be a paradigm for initial events during MELC differentiation.

Abbreviations

aa	amino acid
A	adenine
Ac	acetate
AcHA	6-acatamidohexanoic acid
Ad	adenovirus
AmHA	E-Amino-n-caproic acid
Amp	ampicillin
ATP	adenosine-5'-triphosphate
bp	base pairs
BHK	baby hamster kidney
BSA	bovine serum albumin
C	cytosine
CAT	chloroamphenicol acetyl transferase
cav	cell associated virus
Ci	Curies
CLB	cell lysis buffer
CMV	cytomegalovirus
cpe	cytopathic effect
cpm	counts per minute
crv	cell released virus
CTP	cytidine-5'-triphosphate
Da	Daltons
DAH	1,6-hexanediamine
dATP	2'-deoxyadenosine-5'-triphosphate
DBP	DNA binding protein
dCTP	2'-deoxycytidine-5'-triphosphate
del	deletion
dGTP	2'-deoxyguanosine-5'-triphosphate
DMEM	Dulbecco modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphate
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
dUTP	2'-deoxyuridine-5'-triphosphate

E	early
EBV	Epstein Barr Virus
E.coli	*Escherichia coli*
EDTA	sodium ethylendiamine tetra-acetic acid
EHV	equine herpes virus
EtBr	ethidium bromide
EtOH	ethanol
g	grams
G	guanine
GMEM	Glasgow modification of Eagle Medium
gp	glycoprotein
h	hour(s)
HAT	hypoxanthine, aminopterin, thymidine
HCMV	human cytomegalovirus
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HMBA	N,N'-hexamethylene bisacetamide
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IE	immediate early
IEC	immediate early complex
IR	internal repeat
k	kilodalton
kb	kilobase
l	litre
L	late
LFP	large (Klenow) fragment DNA polymerase
M	molar
min	minute(s)
ml	millilitre
MLP	major late promoter
mM	millimolar
moi	multiplicity of infection
mRNA	messenger RNA
N	unspecified nucleotide or amino acid
NP40	Nonidet P40
OD	optical density
ORF	open reading frame
ORI	origin of DNA replication

^{32}P	phosphorous-32 radioisotope
p.a.	post adsorption
PBS	phosphate buffered saline
pfu	plaque forming unit
PKC	protein kinase C
PKM	protein kinase M fragment
PMSF	phenylmethylsulphonyl flouride
poly(A)	polyadenylic acid
R	purine moiety
RE	restriction enzyme
rRNA	ribosomal RNA
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second(s)
syn	syncytial plaque morphology locus (*syn*$^+$= non syncyti al *syn*= syncyti al)
T	thymidine
TCA	trichloroacetic acid
TEMED	n,n,n'n'-tetramethylethylene diamine
TK	thymidine kinase
TLC	thin layer chromatography
TR	terminal repeat
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
ts	temperature sensitive
TS	thymidylate synthetase
UV	ultra violet
V	volt
vol	volume
v/v	volume/volume
VZV	varicella zoster virus
w/v	weight/volume
WT	wild type
Y	pyrimidine moiety

1. INTRODUCTION

1.1. The Herpesviruses

1.1.1 The *Herpesviridae*

Membership of the family *Herpesviridae* is defined by the structural architecture of the virion and by a number of specific biological properties. The unifying characteristics are listed below, and have been described in all herpesviruses examined to date (For detailed review see Roizman, 1990).

(1) Herpesviruses specify enzymes and other factors involved in nucleoside and nucleotide metabolism.

(2) Viral DNA synthesis and capsid assembly takes place in the nucleus.

(3) Virus replication leads to the destruction of the infected cell.

(4) Herpesviruses are capable of remaining in a latent state in the host cell for the lifetime of the host.

The virions of different members of the *herpesviridae* family cannot be distinguished by electron microscopic examination. However, they are readily identifiable by certain biological properties, virion immunological specificity and by genome size, arrangement and base composition. The family *herpesviridae* is composed of three subfamilies, namely *alphaherpesvirinae*, *betaherpesvirinae* and *gammaherpesvirinae* (Section 1.1.3).

1.1.2 Structural components of Herpes Simplex Virus (HSV) virions

A typical HSV virion consists of a core, a capsid, a tegument and an envelope. The fibrillar core contains a linear double stranded DNA molecule (Wildy *et al.*,1960; Furlong *et al.*, 1972.) within an icosahedral capsid, approximately 100-110nm in diameter. The capsid contains 12 pentameric and 150 hexameric capsomeres which show 5:3:1 axial symmetry (Wildy *et al.*, 1960; McCombs *et al.*, 1971). Schrag *et al.*(1989) produced the first three-dimensional structure of both full and empty HSV-1 capsids, using low dose cryo-electron microscopy in conjunction with computer image analysis. This study deduced that the nucleocapsid was arranged into three distinct structural layers that they designated (i) the outer shell comprising six-coordinated capsomeres which appear hexagonal, (ii) an intermediate

proteinaceous layer with an underlying lattice displaying T=4 icosahedral symmetry, and (iii) an inner shell containing the viral DNA.

In addition to protein and DNA, the HSV-1 nucleocapsid contains spermine which is thought to neutralize some of the negative charge on the DNA and hence facilitate dense packaging of DNA into virions (Roizman and Furlong, 1974).

The capsid is surrounded by an amorphous, asymmetrical material designated the tegument (Roizman & Furlong, 1974). This structure is genetically determined by the virus (McCombs *et al.*, 1971), but the amount of the tegument material can differ, even in the same infected cell (Furlong *et al.*, 1972). The final outermost component is the viral membrane or envelope, which is probably derived from the host cell's nuclear membrane. This triple layered structure includes viral glycoprotein spikes projecting from the surface (Morgan *et al.*, 1959; Epstein, 1962; Hamparian *et al.*, 1963; Morgan *et al.*, 1968; Asher *et al.*, 1969; Spears & Roizman, 1972; Stannard *et al.*, 1987.).

1.1.3 Taxonomy of Herpesviruses

The classification of the *herpesviridae* family into three subfamilies, namely *alpha-*, *beta-*, and *gammaherpesvirinae*, was decided by the Herpes Study Group appointed by the International Committee on Taxonomy of Viruses. These groupings are based on the criteria of host range, length of reproductive cycle, cytopathology and characteristics of latent infection (Roizman *et al.*, 1981).

The neurotropic viruses HSV-1, HSV-2 and VZV are all members of *alphaherpesvirinae*. They are typified by a variable host range *in vitro* and a relatively short reproductive cycle which results in the rapid spread of infection in cell culture with efficient destruction of susceptible cells. *Alphaherpesviruses* have the capacity to establish latent infections that are frequently, but not exclusively, in sensory ganglia.

Betaherpesvirinae, such as HCMV, show a narrow host range *in vivo*, and *in vitro* these viruses replicate most efficiently in fibroblast cells of the appropriate host. This subfamily is also characterised by a relatively long reproductive cycle, with only a slow spread of infection from cell to cell in tissue culture. Frequently, such infected cell are enlarged and form cytomegalia. Latent infections have been described in salivary glands, lymphoreticular cells, kidneys and other tissues.

During infection *in vivo*, members of the *gammaherpesvirinae*, such as EBV, are restricted to replication in the same family or order as the

host. *In vitro*, these viruses infect lymphoblastoid cells and, with exceptions, appear to be specific for either B or T lymphocytes. Lymphotrophic infection is frequently associated with the lack of production of infectious progeny virus. As expected, the lymphoid tissue has been identified as the site of latent infection.

It is now increasingly evident that the classification of herpesviruses on the basis of their biological properties may not accurately reflect their phylogenetic relationships. For example sequence analysis of HHV-6 (Salahuddin *et al.*, 1986) suggests that this virus is more closely related to betaherpesvirinae, (Lawrence *et al.*, 1990) while its host range resembles that of *gammaherpesvirinae*.

1.1.4 Epidemiology of Herpesviruses

In most cases, primary infection with HSV-1 occurs early in life, and any associated disease is frequently indistinguishable from other childhood illnesses. After the initial infection, HSV-1 is retained in a latent form in the neurons of the sensory ganglia (Baringer and Swoveland, 1973; Fraser *et al.*, 1981). Clinical symptoms are not apparent during long term latent infection. Various factors, such as stress, fatigue and exposure to sunlight, are involved in triggering the reactivation of latent virus and the subsequent recurrence of lesions.

A cold sore lesion on the mouth or lips is due to HSV-1 infection (herpes labialis), whereas HSV-1 infection of the corneal epithelia results in herpes keratoconjunctivitus, which in severe cases may lead to blindness. HSV-1 is also associated with skin infections (herpes herpeticum) and with a broad spectrum of neonatal infections. The most devastating HSV-1 infection is herpes simplex encephalitis (Whitley, 1985). Immune compromised patients and renal and cardiac recipients are at particular risk for increased severity of HSV infection (Logan *et al.*, 1971, Muller *et al.*, 1972, Pass *et al.*, 1978).

HSV-2 infection is associated with genital lesions (herpes genitalis) and is usually sexually transmitted, although infection may occur at birth (Whitley, 1985). A small number of cases of genital herpes are associated with HSV-1 infection, which are clinically less severe and less prone to recurrences than HSV-2 infection.

Varicella zoster virus (VZV) is the causative agent of the childhood disease varicella (chicken pox). Infection is spread via the respiratory route and through direct contact, resulting in an irritable skin rash. Shingles

* Human herpes virus type 7 (HHV-7) was isolated by Frenkel *et al.* (1990) in an HIV-1 study, from a culture of CD4+ T cells obtained from a healthy individual. The use of monoclonal antibodies and western blot analysis has demonstrated that HHV-6 and HHV-7 are antigenically distinct (Wyatt *et al.*, 1991). Subsequently, HHV-7 has been frequently isolated from the saliva of healthy individuals (Wyatt & Frenkel, 1992).

(herpes zoster) is associated with a painful rash of dermatomal distribution which results from the reactivation of latent VZV from the sensory ganglia (Hope-Simpson, 1965; Vafai *et al.*, 1988). Both the incidence and the degree of severity of shingles increases with age and in the immunocompromised (Schimpff *et al.*, 1977; Kennedy, 1987).

Infection with the Epstein Barr Virus (EBV) is often asymptomatic with benign disease in children. However, if the time of initial infection is delayed until adolescence, then EBV infection may manifest itself as infectious mononucleosis (glandular fever). EBV is associated with the development of Burkitt's Lymphoma and is linked to a high incidence of nasopharyngeal carcinoma (Epstein and Achong, 1986).

Human cytomegalovirus (HCMV) infections are normally asymptomatic with an increased antibody titre the only evidence of infection. HCMV establishes latency in a number of cell types implicated in the transmission of the virus such as blood, kidney and in host defence cells, for example macrophages (Huang *et al.*, 1978). HCMV is the major cause of viral congenital abnormalities in newborns (Hamilton, 1982). HCMV infection may result in the development of a syndrome resembling EBV infectious mononucleosis.

Human herpesvirus 6 (HHV-6) was originally isolated from patients with AIDS and lymphoproliferative disorders (Salahuddin *et al.*, 1986). It has since been isolated from the saliva of normal adults (Harnett *et al.*, 1990; Levy *et al.*, 1990) and it is clear that 80-90% of the population develop antibodies to HHV-6 early in life (Saxinger *et al.*, 1988; Okunu *et al.*, 1989). HHV-6 has been isolated from children suffering from roseola infantum (exanthum subitum) and there is evidence that this is the causative agent of the disease (Yamanishi *et al.*, 1988). It is not yet clear whether HHV-6 is responsible for any other human diseases and its role in human biology remains largely undefined.

1.1.5 Genome structures of human Herpesviruses

All human herpesviruses have large double stranded linear DNA genomes, the structural features of which are illustrated in Figure 1.1.The complete nucleotide sequence of HSV-1 has been determined (McGeoch *et al.*, 1985, 1986, 1988; Perry & McGeoch, 1988) and comprises approximately 152kbp with a molecular weight of approximately 100 x 10^3 kDal (Becker *et al.*, 1968; Kieff *et al.*, 1971). HSV-1 has a high G+C base composition of 68.3%. However,

Figure 1.1 Gross structures of HSV genomes

The genome structures of the six known human herpesvirus are presented diagramatically as linear molecules. The unique sequences and repeat elements are represented as bold lines and open boxes respectively. Arrows indicate the relative sequence orientation.

The abbreviations employed in the diagram are listed below:-

U_L	unique long
U_S	unique short
I/TR_L	internal/terminal long repeat
I/TR_S	internal/terminal short repeat
D_L	direct repeat left
D_R	direct repeat right
MIR	major internal repeat
ori	origin of DNA replication

This figure was reproduced from McGeoch (1989).

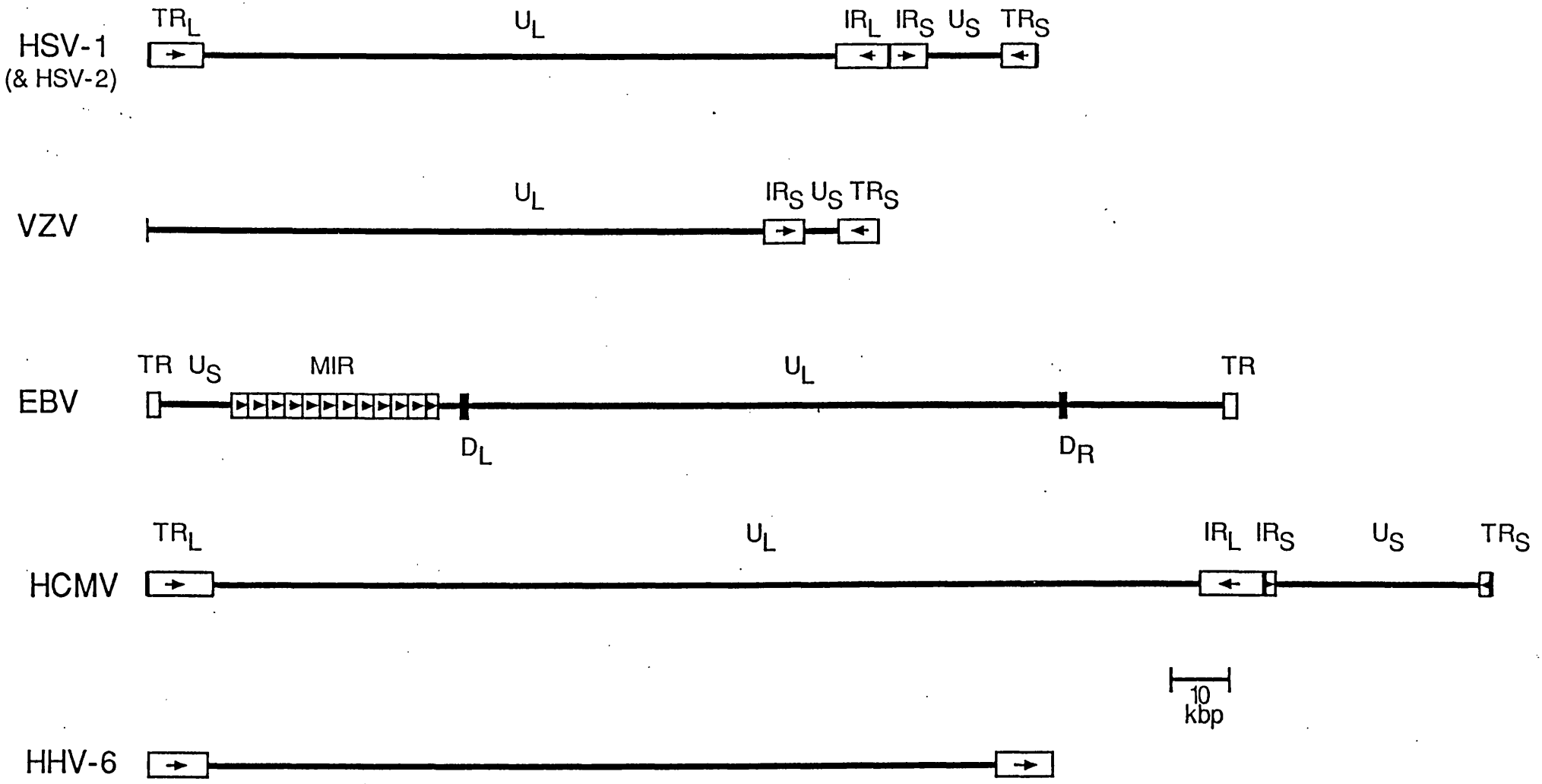

HSV-1
(& HSV-2)
TRL
UL
IRL
IRS
US
TRS
VZV
UL
IRS
US
TRS
EBV
TR
US
MIR
UL
TR
DL
DR
HCMV
TRL
UL
IRL
IRS
US
TRS
10
kbp
HHV-6

Figure 1.1

this value is not constant throughout the genome, with a G+C content of 79.5% in the R_S elements.

Electron microscopic studies have shown that the HSV-1 genome consists of two covalently linked segments, the long region (L) and short region (S). The L segment comprises an unique sequence (U_L) bounded by a pair of oppositely orientated repeat elements (R_L). The sequence elements present in the left terminus (TR_L) designated a,b, are complementary to the internal inverted repeat (TR_L), namely b',a'. The same nomenclature is applied to the S region, which therefore consists of IR_S, U_S and TR_S (Sheldrick & Berthelot, 1974; Figure 1.2).

Based on open reading frame (ORF) predictions, the HSV-1 genome is estimated to encode 74 unique genes, two of which are repeated. 58 genes reside in the U_L region, 12 in U_S, one in each of TR_I and TR_R and one in each of TR_S and IR_S (McGeoch *et al.*, 1988; Figure 1.3). Ackerman *et al.*, 1986; Chou & Roizman, 1990; Barker & Roizman, 1992)

The inverted repeats, R_L and R_S, are approximately 9200bp and 6600bp in size, respectively, (McGeoch *et al.*, 1988). R_L and R_S share the 400bp a sequence (Davison & Wilkie, 1981) which is the minimum terminal repetitive sequence required for optimal circularization (Wadsworth *et al.*, 1976). One or more copies of the 'a' sequence are located internally at the joint between the long and short segments in the opposite orientation to the terminal a sequences (Sheldrick & Berthelot, 1974; Wadsworth *et al.*, 1976; Wagner & Summers, 1978). An overhanging residue, with a free 3' hydroxyl group, is found at the ends of the terminal a sequences (Mocarski & Roizman, 1982).

Preparations of HSV-1 DNA consist of equimolar quantities of four sequence orientation isomers, with U_L and U_S lying independently in one of two possible orientations with respect to the joint between the L and S segments (Delius & Clements, 1976; Wilkie, 1976). One particular isomer has been designated as the prototype orientation to enable standardization of genetic maps (Roizman, 1979; McGeoch, 1989). Evidence is available to support the notion that all four isomers result in both the production of infectious progeny (Davison & Wilkie, 1983) and the development of latent infection (Efstathiou *et al.*, 1986).

To date, approaching 33kbp of HSV-2 DNA sequence has been determined, with all of the U_S region known (McGeoch *et al.*, 1987). HSV-2 is closely related to HSV-1, their genome sequences being closely co-linear (Davison & Wilkie, 1983) and the coding sequences of corresponding genes showing 70-80% nucleotide homology (McGeoch *et al.*, 1987).

Figure 1.2 The positions of the a, b, and c sequences within the HSV genome

The positions of the a, b, and c sequences are indicated. The a', b' and c' sequences are identical to, but in the opposite orientation from, the a, b and c sequences. The number of a sequences at the junction between the L and S components of the HSV genome is variable, but often equal to one.

Figure 1.2

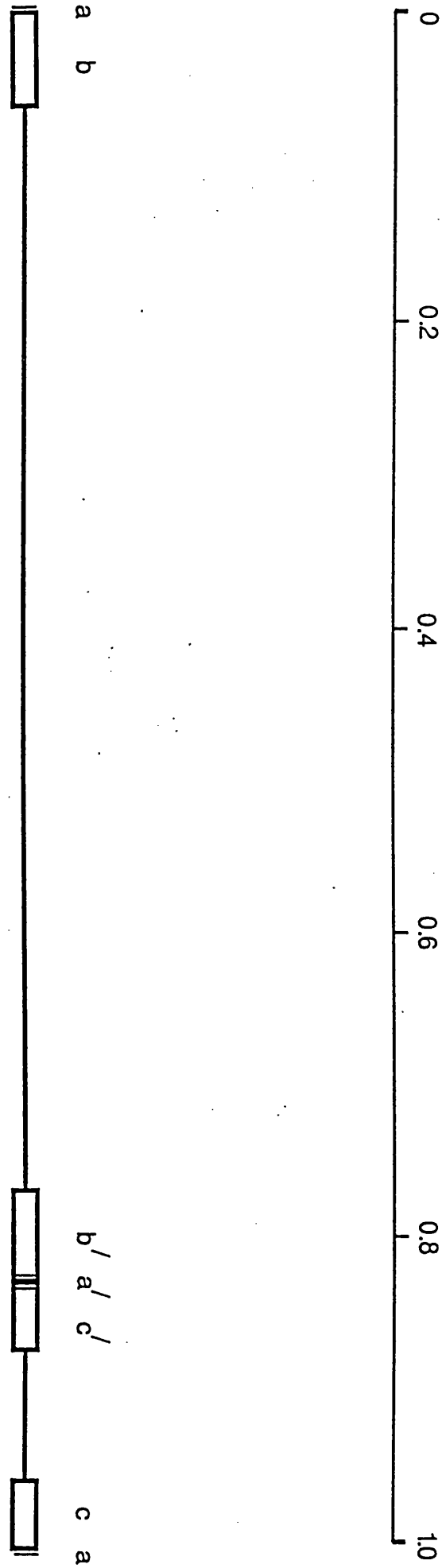


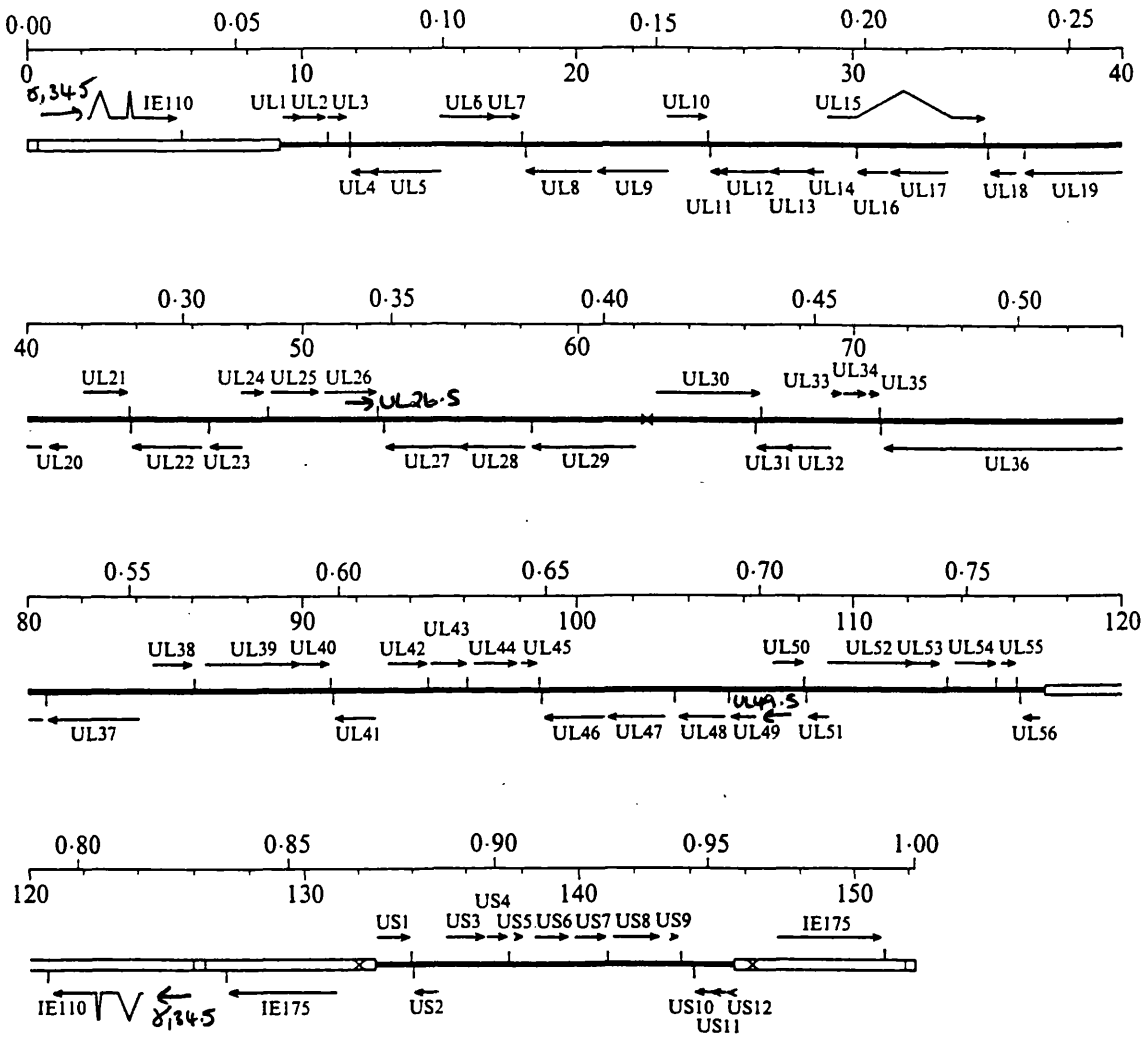
Figure 1.3 Organisation of HSV-1 genes

The locations of the predicted HSV-1 ORFs are shown as arrows, and the corresponding proposed polyadenylation sites are denoted with vertical bars. The upper and lower scales represent map units and kilobase pairs respectively. DNA origins of replications are indicated by an X (Stow, 1982; Spaete & Frenkel, 1982; Stow & McMonagle, 1983; Weller *et al.*, 1985).

This figure was reproduced from McGeoch *et al.* (1988).

(Ackerman *et al.*, 1986; Chou & Roizman, 1990; Barker & Roizman, 1992)

Figure 1.3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UL6 UL7
UL10
UL15
UL4 UL5
UL8 UL9
UL11 UL12 UL13 UL14 UL16 UL17
UL18 UL19
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70
UL21
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UL41
UL46 UL47 UL48 UL49 UL51
UL56
0·80
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0·90
0·95
1·00
120
130
140
150
US4
US1
US3 US5 US6 US7 US8 US9
IE175
IE110
8,34·5
IE175
US2
US10 US12
US11

The entire sequence of the 125kbp genome of VZV is known (Davison & Scott, 1986). The VZV genome has a molecular weight of 80 x 10^3 kDal, with a G+C content of 46%, both of which are lower than the equivalent values for HSV-1. The gross structure resembles that of HSV-1, with the exception of the size of the repeats flanking U_L which is only 88bp in VZV compared to 9200bp in HSV-1. VZV also exists in four isomeric forms, however two of these, representing one orientation of U_L, are 20 times more abundant than the other forms (Davison, 1984).

The first herpesvirus to be entirely sequenced was the B95-8 strain of EBV (Baer *et al.*, 1984) with a genome size of approximately 172kbp and a G+C content of 59.9%. Further investigation revealed that this strain of EBV contains a 13.6kbp deletion, therefore 186kbp is more likely to represent the true size (Raab-Traub, 1980). The organisation of the repeated elements in EBV varies greatly from that of HSV-1 (Fig. 1.1). A direct repeat of a 540bp sequence comprises the termini. Internally, the genome carries a set of 3072bp directly repeated elements, the major internal repeat (MIR), which separates the U_S and U_L. D_R and D_L are almost identical 1000bp repeat elements flanking the U_L segment.

The HCMV genome, the largest of the human herpesvirus, is almost 230kbp with a molecular weight of 147 x 10^6 kDal (Geelen *et al.*, 1978) and a G+C content of 56%. The DNA sequence of the strain AD169 has been determined (Chee *et al.*, 1990) and with respect to the layout of repeat and unique sequences its overall structure is similar to that of HSV-1 (Kilpatrick & Huang, 1977; Westrate *et al.*, 1980). Like HSV-1, the HCMV genome has equimolar amounts of four orientation isomers (Kilpatrick *et al.*, 1976) and an equivalent of the HSV-1 a sequence is also present (Spaete & Mocarski, 1985).

The HHV-6 genome is approximately 160kbp in with a base composition of 41% G+C. It consists of a single unique sequence bounded by a set of large direct repeats and shows some sequence homology with a region of the HCMV genome (Efstathiou *et al.*, 1988).

1.2 Herpes Simplex Virus Lytic Cycle

1.2.1 Initial steps in HSV infection

HSV replication commences with the adsorption of the virion to the cell, followed by penetration through the host membrane. WuDunn & Spear (1989) demonstrated that the initial interaction between both HSV-1 and HSV-2 and the cell membrane is the binding to cell surface heparan sulphate moieties. Agents which block this interaction, or enzymatic digestion of heparan sulphate, also effectively prevent viral adsorption and penetration. There is a requirement for multiple virion-cell surface interactions to occur subsequent to adsorption, to allow fusion of the virion to the cell to commence. Recently Tufaro *et al.* (Abstract, 17th International Herpesvirus Workshop, 1992) demonstrated that a cell line defective in synthesizing the carbohydrate chains of heparan sulphate proteoglycans is actually permissive for HSV infection, thus indicating that other cell surface molecules may substitute for this function.

HSV penetration can occur by both the fusion of the viral envelope with the cell membrane (Epstein *et al.*, 1964; Morgan *et al.*, 1968; Smith & DeHarven, 1974) and by endocytosis (Hummeler *et al.*, 1969; Smith & DeHarven, 1974; Dales & Silverberg, 1969). However, the predominant pathway for productive entry of HSV-1 is by fusion at the cell membrane (Rosenthal *et al.*, 1989). The study by Rosenthal *et al.* (1989) also demonstrated that the penetration of HSV-1 KOS was 100 fold less likely to occur at mildly acidic conditions (pH6.3) than at physiological pH (pH7.4). The relevence of this observation is that electron microscopy studies previously demonstrated that fusion predominates at pH7.4, and endocytosis at pH6.3. In addition mildly acidic conditions have also been shown to inhibit HSV-induced cell to cell fusion and syncytium formation (Lancz & Bradstreat, 1976).

The envelope glycoproteins gB, gD and gH play essential roles in viral penetration (Cai *et al.*, 1987, 1988; Desai *et al.*, 1988; Ligas *et al.*, 1988; Little *et al.*, 1981). Neutralizing monoclonal antibodies raised against gB, gD and gH individually block viral penetration, but have a negligible effect on virion adsorption (Fuller *et al.*, 1987; Highlander *et al.*, 1987, 1988). Recently, Hutchinson *et al.* (1992a, 1992b) identified glycoproteins gK and gL, both of which appear to be essential for HSV replication. gC significantly enhances the binding and infectivity for HEp-2 and Vero cells (Herold *et al.*, 1991). The other known glycoproteins (gE, gG, gI) are apparently dispensable for virus

replication in cultured cells (Drapel *et al.*, 1984; Longnecker & Roizman, 1987; Neidhert *et al.*, 1987).

1.2.2 Alteration of host macromolecular synthesis

The sequential expression of HSV genes during lytic infection usually occurs against a declining background of host macromolecular synthesis (Roizman *et al.*, 1965; Fenwick, 1984). This inhibition of cellular DNA, RNA and protein synthesis, or "host shut-off", is a complex multistage process.

The early or primary phase of host shut-off is mediated by a virion component (Nishioka & Silverstein, 1977) responsible for the dissaggregation of cellular polyribosomes (Sy diskis & Roizman, 1966, 1967) and the subsequent degradation of host mRNAs (Schek & Bachenheimer, 1985). The effects of early host shut-off are seen in the presence of actinomycin (Fenwick & Walker, 1978) and with UV-irradiated HSV-1 (Nishioka & Silverstein, 1977), thus indicating that a virion component is responsible for the phenomenon. The early shut-off is followed by delayed or late shut-off which is conditional upon the synthesis of viral RNA and proteins. Late shut-off is responsible for the enhanced reduction of host protein synthesis and for a decrease in cellular DNA synthesis (Fenwick, 1984).

A range of mutants deficient in host shutoff (*vhs* mutants) have been isolated and characterised (Read & Frenkel, 1983; Kwong & Frenkel, 1987) and have proved important for studying shut-off. *vhs* 1 is defective in early shutoff and consequently fails to degrade pre-existing mRNAs in the absence of viral gene expression. In contrast *vhs* mutants are not defective in late shutoff. The presence of the *vhs* 1 mutation dramatically increases the functional half-lives of viral mRNAs representing all kinetic classes (Oroskar & Read, 1989). The *vhs* gene product appears to destabilise mRNAs with little or no apparent sequence specificity but the mechanism of this *vhs* induced mRNA turnover is not yet known. It has been proposed that the *vhs* gene may either encode a RNase or activate a pre-existing cellular nuclease. Alternatively, the *vhs* function may act by modifying the translational machinery thus rendering the associated mRNA more susceptible to nuclease attack (Oroskar & Read, 1989). The *vhs* 1 mutation has been mapped to a fragment spanning map coordinates 0.604 to 0.606, which contains the UL41 open reading frame, and this gene product is responsible for the early suppression of protein synthesis (Morse *et al.*, 1978; Kwong *et al.*, 1988; McGeoch *et al.*, 1988). UL41 is not an essential structural

component of the virion because viruses containing insertions or deletions in UL41 are viable (Fenwick & Everett, 1990). Several lines of evidence recently demonstrated that the virion transactivator Vmw65 binds to the *vhs* gene product (Weinheimer *et al.*, Abstract, 17th International Herpesvirus Workshop, 1992). Subsequent mutational analysis revealed that *vhs* residues 178-344 were sufficient for complex formation, and that the acidic transcriptional activation domain of Vmw65 was not required. The authors proposed that newly synthesized Vmw65 serves to sequester or dampen the activity of the *vhs* protein produced during the late phase of the lytic cycle (Weinheimer *et al.*, Abstract, 17th International Herpesvirus Workshop, 1992).

Godowski & Knipe (1985) proposed that the production of a viral protein, possibly ICP8, early in infection is responsible for the protection of viral mRNAs from the effects of the virion-associated UL41 gene product. This study demonstrated that infection with the ICP8 ts mutant *ts*13, at the non-permissive temperature, led to a substantially lower level of ICP4 (Vmw175) mRNA accumulation than detected during infection with wild type HSV-1 (KOS). It is therefore possible that ICP8 may function as a mRNA stabilizing factor counteracting the effect of UL41 as ICP8 has a strong affinity for both single stranded and polyA$^+$ RNA (Ruyechan & Weir, 1984) and has previously been shown to downregulate the expression of other viral genes (Godowski & Knipe, 1985).

Not all cellular genes are downregulated during viral infection. Histone gene expression is not repressed by HSV infection (Mayman & Nishioka, 1985), while the abundance of cellular heat shock proteins actually increases during HSV infection (La Thangue *et al.*, 1984; Patel *et al.*, 1985; Kemp *et al.*, 1986). A recent study of three 70,000 molecular weight heat shock genes has shown that only *hsp*70 is induced by HSV-1 infection (Phillips *et al.*, 1991). This specific transcriptional activation of *hsp*70 in HeLa cells is transient and followed by a rapid repression (Phillips *et al.*, 1991). Notarianni and Preston (1982) demonstrated that the overproduction of immediate early polypeptides during *ts*K infection at the non-permissive temperature led to increased synthesis of host heat shock proteins, and further studies showed that it is the overproduction of the mutant Vmw175 polypeptide that induces the stress response (Russell *et al.*, 1987b). The abolition of Vmw63 function during HSV-1 infection prevents the accumulation of a 40kd cellular protein. Vmw63 acts in conjunction with another as yet unspecified factor present in the infected cell (Esteridge *et al.*, 1989). Infection of HFL cells with a temperature sensitive mutant, *ts*1204, deficient in penetration at the non-

permissive temperature, induces the synthesis of a 56kd cellular protein. p56 induction is not detected during a wild type HSV-1 infection, where it is probably inhibited by the expression of an early viral protein (Preston, 1990).

1.2.3 Transcriptional features of HSV-1 genes

HSV-1 genes are transcribed by cellular RNA polymerase II, in the nucleus of the host cell (Alwine *et al.*, 1974; Ben-Zeev & Becker, 1977; Costanzo *et al.*, 1977). The resultant viral mRNAs share common structural features with cellular RNAs. They have a 5' cap structure, are polyadenylated at the 3' end, and are internally methylated (Bachenheimer & Roizman, 1972; Silverstein *et al.*, 1973; Bartoski & Roizman, 1976). The occurrence of 3' co-terminal families, where a particular gene has its own promoter and unique 5' terminal sequences, but shares 3' terminal sequences with other transcripts, is common. This arrangement is seen in the U_S where 11 of the 13 mRNAs (encoding 12 proteins) are arranged into four nested families (Rixon & McGeoch, 1984, 1985; McGeoch *et al.*, 1986.). Overlapping reading frames and 5' ends on opposite strands are also observed, albeit relatively rarely (Rixon & McGeoch, 1984; Wilkie *et al.*, 1980). Spliced transcripts are also a minority feature of HSV-1 transcription. Out of the 72 proposed distinct genes (Fig. 1.2), only the IE-1 (Perry *et al.*, 1986), IE-4/5 (Watson *et al.*, 1981; Rixon & Clements, 1982), UL15 (Costa *et al.*, 1985; McGeoch *et al.*, 1988) and the latency associated transcripts (LATs) (Wagner *et al.*, 1988; Wescher *et al.*, 1988) are known to generate mature mRNA by splicing.

HSV-1 gene expression occurs in a coordinated temporal manner, with HSV transcription divided into three distinct phases, namely IE (α), E (β) and L (γ) (Swanstrom & Wagner, 1974; Jones & Roizman, 1979; Clements *et al.*, 1977). The IE genes are the first class to be transcribed immediately upon entry into the host cell and in the absence of *de novo* protein synthesis (Kozak & Roizman, 1974; Clements *et al.*, 1977; Jones & Roizman, 1979). IE polypeptides play regulatory roles in HSV gene expression, and the properties of the five IE proteins are described in section 1.2.5. Subsequent expression of early genes which encode proteins involved in DNA replication and nucleotide metabolism is dependent upon functional IE gene products. Vmw63 appears to be involved in the switch from early to late gene expression (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice & Knipe, 1990). Late gene expression is split into two different stages, early late (γ_1) and "true" late (γ_2) with the distinction that transcription of γ_2 genes occurs only after

viral DNA replication has taken place (Jones & Roizman, 1979; Holland *et al.*, 1980; Hall *et al.*, 1982; Godowski & Knipe, 1985; Johnson *et al.*, 1986). In contrast, γ_1 proteins are produced at detectable levels when DNA synthesis is inhibited. Late genes generally encode structural proteins and proteins involved in the production of progeny virions.

The expression of HSV-1 genes is primarily controlled at the level of transcription initiation. However, McLauchlan *et al.* (1989) demonstrated that an HSV-induced factor regulates polyadenylation site usage. The presence of this heat labile factor selectively increases cleavage at a late polyadenylation site *in vitro*. However, sequence comparison of polyadenylation sites representing all temporal classes of HSV genes does not reveal any consensus sequence associated specifically with late polyadenylation sites (McLauchlan *et al.*, 1989).

Temporal regulation of the three classes of HSV genes is governed by the 5' flanking sequences associated with the different promoters classes. Sequence analysis of upstream promoter regions demonstrated that they contained *cis*-acting elements recognised by both viral and cellular transacting factors.

1.2.4 Immediate-early gene expression

The nature of the regulatory sequences associated with immediate early (IE) gene expression was originally investigated by Post *et al.* (1981). Their paper describes the conversion of TK^- viruses to a TK^+ phenotype in which structural sequences of the HSV thymidine kinase gene were linked to either its own promoter or to the 5' flanking sequences constituting the IE-3 promoter. Cells infected with the IE-3-TK recombinant virus under immediate early conditions showed induced TK activity upon the release of the cycloheximide block. In contrast, the wild type virus did not express thymidine kinase in the absence of protein synthesis. The IE-3 5' terminal sequences are sufficient to confer IE- regulatory characteristics upon the normally early TK gene, and the positioning of these sequences at the 3' end of the promoter abolished the expression of TK (Post *et al.*, 1981). A TK^+ transformed cell line was also constructed with the TK gene under the control of the IE-3 promoter. Superinfection with a TK^- virus resulted in the stimulation of TK activity from the IE-3 promoter, even in the presence of cycloheximide. A similar induction was observed at both permissive and non-permissive temperatures by the superinfection of a *ts* virus (*ts*502-Δ305) that lacks a functional Vmw175. Post *et al.* (1981) suggested that enhancement

from the IE-3-TK chimera was due to a virion component, provided by the superinfecting TK^- virus, which did not depend on IE gene products. Similar results were later obtained using TK^+ cell lines derived from IE-1-TK and IE-3-TK chimeras (Mackem & Roizman, 1982a, 1982b).

UV-irradiated TK^- virus was shown to be as effective as untreated virus at inducing TK activity from IE-3-TK chimeras (Batterson & Roizman, 1983). A temperature sensitive mutant, *ts*B7, defective in uncoating, stimulated IE gene expression at the non-permissive temperature, at which viral DNA is not released from the capsid, indicating that the factor responsible for induction of IE genes is a component located outwith the nucleocapsid (Batterson & Roizman, 1983). This virion component was later identified by Campbell *et al.* (1984) in a series of co-transfection experiments in which cloned restriction fragments of HSV and an IE-TK reporter plasmid were introduced into cells and the resultant TK activity was determined. The *Bam*HIf fragment (0.64 to 0.69 map units) was found to encode an activity which stimulates only IE-TK activity. This transactivation activity was subsequently localised to a 2.3kbp subclone of the *Bam*HIf fragment. Hall *et al.* (1982) showed that a major 1.9kb mRNA species was the only entire transcript originating from the region encompassed by this subclone. Hybrid-arrested translation, in conjunction with immunoprecipitation analyses, were employed to verify that this transactivating virion component was indeed Vmw65 (Campbell *et al.*, 1984).

The product of the UL48 gene, Vmw65 (also known as VP16), is the major tegument protein, and it is estimated that there are approximately 1000 molecules per virion (Heine *et al.*, 1974; Roizman & Furlong, 1974). Vmw65 is essential for the assembly of progeny virions, since the HSV-2 mutant *ts* 2203, whose mutation maps to Vmw65 coding sequences, fails to assemble virus particles at the non-permissive temperature (Ace *et al.*, 1988).

The sequence elements located in the 5' upstream region responsible for mediating the responsiveness to transactivation by Vmw65 were determined by sequence and deletion analyses. A consensus AT-rich cis-acting sequence, TAATGARATTC (R = purine) was identified, the presence of which was integral to Vmw65 responsiveness. (Mackem & Roizman, 1982a,b,c; Cordingley *et al.*, 1983; Kristie & Roizman, 1984; Lang *et al.*, 1984; Preston & Tannahil, 1984; Gaffney *et al.*, 1985; Bzik & Preston, 1986). The TAATGARAT consensus sequence was shown to be present in the far upstream regions of all the HSV-1 IE genes (Murchie & McGeoch, 1982; Mackem & Roizman, 1982a,b; Whitton *et al.*, 1983; Whitton & Clements, 1984) and its activity is also modulated by flanking sequences. In IE4/5 and IE-3, these regions contain

the heptanucleotide CCCGCCC or its complement GGGCGGG (Preston & Tannahill, 1984) which represent binding sites for the transcription factor Sp1 (Jones & Tjian, 1985). Studies by Kristie & Roizman (1984) highlight the importance of similar flanking sequences, the GA-rich motifs identified in the upstream regulatory regions of the IE-2, IE-3 and IE-4/5 promoters. These GA-rich elements do not have any independent activity, but can convert an otherwise non-functional related element, TAACGAGGAAG, to a state of virion responsiveness (Bzik & Preston, 1986).

The IE-3 far upstream region, between nucleotide positions -174 to -332, contains an enhancer-like element (Post *et al.*, 1981; Mackem & Roizman, 1982a; Cordingley *et al.*, 1983; Lang *et al.*, 1984; Preston & Tannahill, 1984) which is not functionally dependent on Vmw65, and can stimulate expression in both orientations and from a considerable distance (>1000bp) (Lang *et al.*, 1984; Preston & Tannahill, 1984). However, in contrast to other characterised enhancers, this element does not appear to function when positioned downstream of the target gene. Both the GC-rich and GA-motifs are integral components of this enhancer (Preston & Tannahill, 1984).

After the discovery that Vmw65 acts through the TAATGARAT element, emphasis moved towards revealing its mode of action. Vmw65 itself does not possess any intrinsic DNA binding activity, neither specifically for the TAATGARAT element nor non-specifically for double stranded DNA (Marsden *et al.*, 1987; Preston *et al.*, 1988). This apparent paradox was resolved when Vmw65 was shown to form a specific complex with the TAATGARAT motif, only in the presence of cellular proteins under conditions that support the binding of these cellular factors to DNA (McKnight *et al.*, 1987; O'Hare & Goding, 1988; Preston *et al.*, 1988). Studies prior to this work had revealed that nuclear extracts from uninfected tissue culture cells contained two major proteins that specifically bound to the TAATGARAT site (Kristie & Roizman, 1987). DNase I footprinting analysis and gel retardation assays using uninfected HeLa nuclear extract, confirmed the formation of a specific complex between cellular proteins and the TAATGARAT motif (O'Hare & Goding, 1988; Preston *et al.*, 1988). This novel complex, denoted the IEC (immediate early complex), was detected using either infected nuclear extract or uninfected extract supplemented with virion extract (Preston *et al.*, 1988).

Monoclonal antibodies, LP1 (McLean *et al.*, 1982) and MA1044 (Campbell *et al.*, 1984), and a third raised against a peptide representing the C-terminus of Vmw65 (Dalrymple *et al.*, 1985; Pellet *et al.*, 1985) specifically decreased the electrophoretic mobility of the IEC (Preston *et al.*, 1988). The addition of affinity purified Vmw65 or *in vitro* synthesized [^{35}S]-methionine

labelled Vmw65 to a HeLa cell nuclear extract correlated with IEC formation (McKnight *et al.*, 1987; Preston *et al.*, 1988) thus confirming that Vmw65 is indeed a component of the IEC.

Several point mutations in the TAATGARAT element, such as TAA to TCC, abolish IEC formation and oligonucleotides containing this mutant sequence also fail to compete with TAATGARAT for IEC formation (O'Hare & Goding, 1988; Preston *et al.*, 1988). When a range of mutant oligonucleotides were individually introduced upstream of a previously non-responsive promoter, a direct correlation between failure to direct IEC formation and the inability to confer Vmw65 responsiveness was established (Preston *et al.*, 1988; O'Hare & Goding, 1988).

Sequence analysis revealed that the TAATGARAT motif is highly homologous to the NFIII binding site in the adenovirus origin of DNA replication (Pruijn *et al.*, 1986), to the octamer consensus element ATGCAAAT (Rosales *et al.*, 1987) located in the immunoglobulin light chain enhancer and promoter (Ephrussi *et al.*, 1985; Falkner *et al.*, 1984) and the human histone H2B promoter (Sive & Roeder, 1986). O'Hare and Goding (1988) initially suggested that the protein Oct-1 (NFIII, OTF-1), present in a wide range of cell types, was one of the cellular components of IEC. Gerster & Roeder (1988) subsequently confirmed that Oct-1 purified from HeLa cells was required for the formation of the tertiary complex IEC (Gerster & Roeder, 1988). Involvement of one or more additional cellular factors was implied as the incubation of *in vitro* synthesized Vmw65 and purified Oct-1 with TAATGARAT did not result in IEC formation. However, subsequent addition of an Oct-1 depleted HeLa cell nuclear extract dramatically restored binding to TAATGARAT (Gerster & Roeder, 1988).

Several transcription factors, such as Oct-1, Pit-1 and *unc*86, involved in tissue-specific and developmental regulation (Bodner *et al.*, 1988; Ingraham *et al.*, 1988; Finney *et al.*, 1988) share a characteristic POU domain comprised of two conserved motifs, the POU-specific domain and the POU homeodomain. Regions of the highly conserved POU domain are homologous to the proposed DNA-binding domain of Drosophila developmentally regulated transcription factors (Ko *et al.*, 1988; Muller *et al.*, 1988; Scheider *et al.*, 1988).

A combination of deletion and insertional mutagenesis revealed that Vmw65 is composed of two distinct and functionally separable domains (Sadowski *et al.*, 1988). The C-terminal 78 amino acids specify the trans-activation activity whereas the N-terminal region contains the requirements for IEC formation. The C-terminal domain is markedly enriched for

negatively charged amino acid residues. Out of the 78 C-terminal amino acids, 21 have acidic side chains that are either aspartic or glutamic acid residues and none are basic (Dalrymple *et al.*, 1985). This 'acidic tail' is highly reminiscent of the acidic transcriptional activation domains previously identified in the yeast regulatory proteins GCN4 and GAL4 which are involved in the regulation of amino acid biosynthesis and galactose metabolism respectively (Hope & Struhl, 1986; Gill & Ptashne, 1987). The Vmw65 acidic domain functions *in vivo* when coupled to the DNA binding domain of either GAL4 (Sadowski *et al.*, 1988) or to the human oestrogen receptor (Tora *et al.*, 1989). The hybrid GAL4-Vmw65 protein also functions as a transcriptional activator *in vitro* with both yeast (Berger *et al.*, 1990) and HeLa cell extracts (Carey *et al.*, 1990). Using an *in vitro* assay, Berger *et al.* (1990) demonstrated that the chimeric protein selectively inhibited activated but not basal transcription. This inhibitory effect is thought to be due to the sequestering of factors essential for transcriptional activation (Ptashne, 1988). Biochemical evidence has since been provided for a transcriptional intermediary factor (TIF) in HeLa whole cell extracts which is required to mediate the stimulatory activity of the acidic activator GAL4-Vmw65 (White *et al.*, 1991). This factor is distinct from previously identified transcription factors as it is not essential for basal transcription. White *et al.* (1991) propose an association between TIF(s) and TFIID, and suggest that the TIF(s) may be the direct target of the acidic domain of GAL4-Vmw65.

Triezenberg *et al.* (1988b) constructed a series of C-terminal deletions in Vmw65 and demonstrated that the C-terminal 78 amino acids were crucial for transcriptional stimulation. A C-terminal truncated protein, encoding only part of the acidic region, is partially active in transactivation assays. Triezenberg *et al.* (1988a) originally suggested that the overall number of negatively charged residues is important and not necessarily the integrity of the acidic region itself. However, detailed mutagenic analysis of the acidic activation domain has since revealed that although the net charge contributed, it was not sufficient for transcriptional activation by Vmw65 (Cress & Triezenberg, 1991). The phenylalanine residue at position 442 is extremely sensitive to mutation, and is thought to be involved in a hydrophobic interaction (Cress & Triezenberg, 1991).

Using a transient expression system in conjunction with N-terminal deletion mutants, Greaves & O'Hare (1990) demonstrated that the N-terminal 403 amino acids are essential for IEC formation. A truncated form of Vmw65, specifying only the N-terminal portion and therefore lacking the acidic tail, dominantly interfered with the wild type activator in transient transfection

McKnight *et al.* (1986, 1987) identified two factors, namely the products of UL46 and UL47, that enhance the efficiency of Vmw65 dependent activation of IE gene expression. Subsequent work using viral deletion mutants demonstrated that neither UL46 nor UL47 encoded gene products, either separately or in combination, were required for viral growth in tissue culture (Zhang *et al.*, 1991). A UL47 deletion mutant resulted in an 80% reduction in ability to induce the expression of an IE-regulated thymidine kinase gene resident in 143TK$^-$ cells. A UL46 deletion mutant had no effect on the level of Vmw65 synthesized in the infected cell (Zhang *et al.*, 1991). Zhang and McKnight (1993) showed that the UL46 and UL47 viral deletion mutants lacked VP11 and VP12 or VP13 and VP14 respectively. It was proposed that these proteins act at the same level since single mutants were associated with a 2h delay in TK expression while double mutants were associated with a 4h delay in TK expression. The authors predicted that the ability of UL46 and UL47 to enhance transcription resulted from the stochiometric association of VP11/VP12 and VP13/VP14 with Vmw65 in the infecting virion (Zhang and McKnight 1993).

assays (Triezenberg *et al.*, 1988b). Triezenberg *et al.* (1988a) proposed that this N-terminal segment competes with the native protein for the specific interaction sites on the cellular DNA binding proteins. A similar hypothesis was postulated to explain the specific reduction in permissiveness to HSV-1 replication of a transformed cell line expressing a truncated Vmw65 protein lacking the acidic activation domain by HSV-1 at low MOI (Freidman *et al.*, 1988).

Ace *et al.* (1988) constructed a series of 12bp linker insertion mutants throughout the Vmw65 open reading frame. Each mutant plasmid was assayed for its ability to stimulate IE promoters in short term transfection assays; to form IEC in an *in vitro* binding assay; and for the ability to rescue the HSV-2 assembly mutant *ts* 2203. These results demonstrate a direct correlation between ability to form the tertiary complex IEC, and stimulation of IE- gene transcription (Ace *et al.*, 1988). To determine which domains of Vmw65 are involved in the correct assembly of progeny virions, the mutated plasmids were assayed for their ability to rescue *ts* 2203, which fails to assemble virions at the non-permissive temperature. One plasmid, *in*14, rescued *ts* 2203, therefore the insertion had not disrupted a domain crucial for the structural integrity of Vmw65. However, *in*14 failed to form the IEC, demonstrating that Vmw65's functional domains are separable and raising the possibility that viable viruses containing the *in*14 insertion could be constructed (Ace *et al.*, 1988). This was subsequently achieved by Ace *et al.* (1989) who constructed the mutant *in*1814.

The mutant *in*1814 contains a 4 amino acid insertion at codon 379 which disrupts the region of Vmw65 required for IEC formation, but does not interfere with virion assembly (Ace *et al.*, 1988, 1989; Greaves & O'Hare, 1990). This mutation causes a decrease in the levels of IE-RNA accumulation and viral protein synthesis under immediate early conditions. Comparison of BHK cells infected with *in*1814 or HSV-1 in the presence of cycloheximide demonstrated a 4-5 fold reduction in the level of accumulation of IE-1 and IE-2 RNA and a 2 fold decrease in IE-4 RNA, whereas the level of IE-3 RNA was unchanged. Densitometric analysis of [^{35}S]-methionine labelled IE polypeptides revealed that the levels of Vmw110 and Vmw63 polypeptides were also reduced 4-5 fold, and the level of Vmw175 was unaffected. Both of these values are in good agreement with the IE RNA synthesis rates. However, it was not possible to measure the Vmw68 levels accurately because this polypeptide ran as a diffuse band (Ace *et al.*, 1989). *in*1814 has a high particle:pfu ratio (1000:1) which means that at low MOI plaque formation is

Weinheimer *et al.* (1992) recently described the construction of a mutant virus containing a deletion in the Vmw65 open reading frame, such that no functional Vmw65 is produced. This mutant replicated as efficiently as wild type HSV-1 during infection of a Vmw65 expressing transformed cell line, resulting in the production of mutant viruses containing cell-derived Vmw64 recruited during virion assembly. Although this cell derived Vmw65 was functional for IE gene *trans*induction, plaque assays and single step replication assays revealed that the mutant failed to replicate during subsequent infection of cells that do not express Vmw65. This work demonstrates that the absence of Vmw65 at late times during infection precluded the production of infectious progeny virus and correlates with defective HSV-1 particle assembly (Weinheimer *et al.*, 1992).

severely disabled. This high particle:pfu ratio is even more pronounced in HFL cells (Ace *et al.*, 1989; Harris & Preston, 1992).

It appears that the metabolic state of the host cell influences the efficiency of plaque formation, as the apparent titre of a given stock of *in*1814 on different batches of BHK cells can vary by as much as 10 fold (Ace *et al.*, 1989). Preston (unpublished observations) noted that titration of *in*1814 on freshly seeded HFL cells also increases the efficiency of plaque formation. Recently, Daksis & Preston (1992) demonstrated that in the absence of functional Vmw65, HSV-1 immediate early gene expression varies during the cell cycle. They found that *in*1814 formed plaques less efficiently on HeLa cells synchronised at the G2 phase of the cell cycle than at G1/S, demonstrating an increased requirement for Vmw65 when cultures reached the G2 phase.

The reduced ability of *in*1814 to initiate plaque formation at low MOI was partially overcome by either the provision of Vmw110 *in trans* or by prior infection with UV-irradiated *ts*K supplying functional Vmw65 (Ace *et al.*, 1989). The importance of functional Vmw65 in the progression into productive lytic infection was strengthened by Werstuck *et al.* (1990), who showed that transfection of HSV-1 viral DNA into a cell line expressing Vmw65 resulted in enhanced infectivity.

The work by Ace *et al.* (1989) demonstrated that transinduction of IE gene expression by Vmw65 is extremely important at both low MOI in tissue culture systems and *in vivo*, as *in*1814 is essentially avirulent in mice. This *in vivo* situation also emphasizes the importance of host cell factors in the decision between the replication of *in*1814 or the establishment of a latent infection. *in*1814 has been used as a tool to investigate the role of Vmw65 in latent infections, *in vitro* (Harris & Preston, 1992) and *in vivo* (Steiner *et al.*, 1990).*

The presence of hexamethylene bisacetamide (HMBA) in the culture medium has recently been shown to overcome the replication defect of *in*1814 (McFarlane *et al.*, 1992; this thesis). HMBA and DMSO are known to induce differentiation of cultured erythroleukemic cells and to enhance the reactivation of latent HSV after explantation of the ganglia. HMBA increased the level of IE RNA accumulation in the presence of cycloheximide. McFarlane *et al.* (1992) propose that HMBA- and DMSO-mediated enhancement of reactivation from latency is due to an increase in IE RNA production.

1.2.5 Properties and functions of immediate early polypeptides

HSV-1 encodes 5 immediate early genes specifying the polypeptides Vmw110 (ICP0), Vmw63 (ICP27), Vmw175 (ICP4), Vmw68 (ICP22) and Vmw12 (ICP47) (Watson *et al.*, 1979; Anderson *et al.*, 1980).

1.2.5.1 Vmw175

The IE-3 gene, encoding Vmw175, is located in both copies of the short repeat regions and is therefore diploid (Rixon *et al.*, 1982). From SDS polyacrylamide gel electrophoresis the polypeptide product has an estimated molecular weight of 175,000 and consists of 1298 amino acid residues (McGeoch *et al.*, 1986). Vmw175 is a critical immediate early gene product as it is essential for the subsequent expression of both early and late genes and for the repression or autoregulation of immediate early gene expression (Preston, 1979a; Watson & Clements, 1980).

Vmw175 is a nuclear DNA binding protein which is post-translationally modified by phosphorylation at both serine and threonine residues and can be poly(ADP-ribosyl)ated in isolated nuclei (Powell & Purifoy, 1976; Pereira *et al.*, 1977; Cabral *et al.*, 1980; Hay & Hay, 1980; Preston & Notarianni, 1983; Faber & Wilcox, 1986). It is possible that the state of phosphorylation of Vmw175 affects its regulatory activities as pulse-chase experiments indicate that phosphate groups can cycle on and off the protein during infection (Wilcox *et al.*, 1980). It is now known that different electrophoretic forms of Vmw175 differ in their abilities to bind to specific HSV regulatory sequences (Michael *et al.*, 1988). Vmw175 can be isolated in multimeric forms, but normally exists as a homodimer (Metzler & Wilcox, 1985).

Vmw175 can specifically recognize and bind to bipartite sites related to the sequence ATCGTnnnnnCGG (Faber & Wilcox, 1986; Muller, 1987; Kattor-Codey & Wilcox, 1989; Pizer *et al.*, 1991; Everett & Orr, 1991), but this sequence alone is not sufficient for binding (Roberts *et al.*, 1988; Pizer *et al.*, 1991). Indeed, other binding sites have been defined which do not contain the consensus Vmw175 binding site (Beard *et al.*, 1986; Kristie & Roizman, 1986; Michael *et al.*, 1988; Michael & Roizman, 1989; Tedder *et al.*, 1989). The situation is further complicated because consensus Vmw175 binding sites are not commonly found in the promoter regions of many early and late genes

despite the observation that Vmw175 is essential for their activation. The early TK promoter contains weaker, non-consensus, sites (Imbalzano *et al.*, 1990) and it has recently been suggested that transactivation of the TK promoter by Vmw175 does not depend on binding to these sites (Imbalzano *et al.*, 1990; Shepard & DeLuca, 1991). The late gD promoter contains two Vmw175 binding sites 5' of the promoter region, and a third site downstream of the transcriptional start site. In transfection assays, these sites have been shown to contribute to activation (Tedder *et al.*, 1989) and multimerization of any one of these sites is correlated with increased activation by Vmw175 (Tedder *et al.*, 1988). However, Everett (1983, 1984b) demonstrated that the two upstream sites are not essential for transactivation by Vmw175 under conditions providing all the immediate early polypeptides. Recently a mutant virus was constructed harbouring mutations in all three Vmw175 binding sites in the gD promoter (Smiley *et al.*, 1992). This study reveals that these sites are not essential for transcription of gD RNA during virus infection, and led to the proposition that the Vmw175 binding sites are sufficiently numerous or redundant in the viral genome, such that if any particular site is removed, then its role is taken on by another site (Smiley *et al.*, 1992).

Comparison of the predicted amino acid sequence of Vmw175 with related proteins expressed by VZV (McGeoch *et al.*, 1986), PRV (Vlcek *et al.*, 1989) and EHV-1 (Grundy *et al.*, 1989) revealed that Vmw175 is highly conserved and is composed of 5 domains. Regions 2 and 4 show a high degree of conservation while regions 1, 3 and 5 are less related.

Evidence has accumulated supporting the hypothesis that binding of Vmw175 to the viral genome is an important stage in the activation of transcription. The mutant *ts*K has a single amino acid substitution at residue 475 in the DNA binding domain (Davison *et al.*, 1984) and at the NPT, and this abberant Vmw175 polypeptide neither binds DNA nor activates gene expression (Preston, 1979b; Paterson & Everett, 1988).

Insertion and deletion mutants in plasmid-encoded Vmw175 have been studied in DNA binding assays. These experiments demonstrate that the principal part of the polypeptide important for DNA binding is in region 2 (DeLuca & Schaffer, 1988; Paterson & Everett, 1988; Shepard *et al.*, 1989). When these plasmids were co-transfected with a reporter gene construct in transient transfection assays, Paterson & Everett (1988) deduced that both regions 2 and 4 were involved in the regulation of gene expression by Vmw175. The portion of the protein essential for transactivation also has the ability to bind DNA and is highly conserved amongst the other members of the *alphaherpesvirinae* subfamily (McGeoch *et al.*, 1986; Cheung, 1989;

Grundy *et al.*, 1989; Vlcek *et al.*, 1989). The mutation in *ts*K lies in this highly conserved region (Davison *et al.*, 1984).

The product of the VZV gene 62, a 140,000 polypeptide, has been shown to complement the growth of HSV-1 viruses with temperature sensitive lesions and deletions in Vmw175 (Felser *et al.*, 1987, 1988). Based on this functional homology, Disney & Everett (1990) constructed a recombinant virus in which both copies of the Vmw175 coding sequence were replaced by the VZV open reading frame of gene 62 (Davison & Wilkie 1983; McGeoch *et al.*, 1986). The progeny virus, HSV-140, was viable in tissue culture but failed to form plaques. At a high MOI, the expression of certain late genes was reduced, but the majority of the viral polypeptides were synthesized in apparently normal amounts. Analysis of protein synthesis revealed that the VZV 140,000 protein was present in large amounts late in infection, indicating that this polypeptide does not autoregulate the HSV IE-3 promoter (Disney & Everett, 1990). In contrast, Vmw175 represses its own expression through binding to the specific Vmw175 DNA binding sequence at the cap site of the IE-3 promoter (Muller *et al.*, 1987; Roberts *et al.*, 1988). This phenomenon of autoregulation can be reproduced in transient assays (O'Hare & Hayward, 1985b, 1987). *In vitro* analyses have demonstrated that mutants of Vmw175 that fail to bind to the IE-3 cap site also fail to repress expression from the IE-3 promoter (DeLuca & Schaffer, 1988; Paterson & Everett, 1988). It has recently been demonstrated that the activation domain of Vmw175 interacts with the host transcriptional apparatus through direct protein-protein contacts with TFIID and TFIIB (Smith & DeLuca, Abstract, 17th International Herpesvirus Workshop, 1992).

Everett *et al.* (1992) showed that the process of Vmw175 binding to DNA physically bends it to a 60^{o} angle at or very close to its binding site. Binding of both purified Vmw175 DNA binding domain and partially purified preparations of the intact protein induced DNA bending (Everett *et al.*, 1992). The VZV homologue 140,000 protein does not bend DNA but does disrupt the helix (Tyler & Everett, personal communication). This observation may, at least partially explain why the VZV homologue does not entirely functionally substitute for Vmw175 in HSV (Disney & Everett, 1990).

1.2.5.2 Vmw110

The IE-1 gene is located entirely within the long repeat elements (R_L) of the HSV genome, and is therefore diploid (Preston *et al.*, 1978). The primary transcript is spliced to remove 2 introns which, unusually for HSV,

are in polypeptide coding regions (Perry *et al.*, 1986). The latency associated transcripts (LATs) (Section 1.3.2.5) partially overlap and show sequence complementarity with the 3' ends of the IE-1 mRNA (Stevens *et al.*, 1987; Wagner *et al.*, 1988; Wechsler *et al.*, 1988).

The IE-1 gene encodes a 775 amino acid polypeptide product with a predicted molecular weight of 78,452. However, SDS polyacrylamide gel electrophoresis indicated that Vmw110 has an apparent molecular weight of 110,000, as a consequence of modifications. Vmw110 is a nuclear phosphoprotein (Marsden *et al.*, 1976) which binds to DNA in crude cell extracts *in vitro* and is associated with chromatin *in vivo* (Pereira *et al.*, 1977; Hay & Hay, 1980). Analysis of the polypeptide sequence reveals a cysteine rich potential zinc finger binding domain between amino acids 130 and 160. A zinc finger structural motif was originally identified in the transcription factor TFIIIA (Miller *et al.*, 1986) and subsequently in a number of DNA binding proteins which participate in the regulation of transcription (Berg, 1986; Evans & Hollenberg, 1988). Everett *et al.*, (1991) used metal affinity chromatography to demonstrate that Vmw110 in nuclear extracts is retained on zinc columns and that this interaction does not depend on the zinc finger. Removal of the zinc finger abolishes the normal nuclear localization of Vmw110 resulting in a cytoplasmic association. Given that zinc fingers are also involved in the protein:protein interactions required for transactivation of HIV (Frankel *et al.*, 1988), it has been postulated that the function of the zinc finger in Vmw110 is to promote an association with another protein, perhaps through the C-terminal region (Everett *et al.*, 1991). The ability of Vmw110 to bind zinc has recently been confirmed by Vaughan *et al.* (1992).

Vmw110 is a potent and promiscuous transactivator of gene expression in transient transfection assays (Everett, 1984a; O'Hare & Hayward, 1985a; Gelman & Silverstein, 1985; Quinlan & Knipe, 1985). It displays a lack of target specificity, being capable of activating transcription from herpesvirus promoters representing all temporal classes, and from a large number of heterologous promoters such as the HIV LTR (Mosca *et al.*, 1987) and the SV40 early promoter (O'Hare *et al.*, 1986; Everett, 1988). Vmw110 can also act in synergy with Vmw175 to activate transcription (Everett 1984a, 1986) and, in some transfection systems, this synergistic activation can represent a 20 fold increase in activity compared to the levels obtained with either alone (Everett, 1984a).

The functional domains of Vmw110 were investigated by constructing a series of small in-frame insertion or deletion mutants and testing these variants for their ability to transactivate a gD-promoter linked

to a CAT reporter gene in a transient transfection assay, both in the presence and absence of Vmw175 (Everett, 1987a, 1988). 5 separate regions were identified which were required for maximum synergistic activity with Vmw175. The C-terminal rich domain appears to be crucial for function in synergy with Vmw175, but not a requirement for the intrinsic activity of Vmw110 alone. Immunofluorescent staining revealed that the C-terminal amino acids 474 to 509 contained features essential, but not sufficient, for nuclear localization (Perry *et al.*, 1986; Everett, 1986; Kalderon *et al.*, 1984).

To determine whether the functional regions defined by transient transfection assays using plasmid borne Vmw110 had corrresponding consequences in the viral genome, mutant virus with defined lesions in the IE-1 gene were constructed (Stow & Stow, 1986; Everett, 1989). *dl*1403, *dl* X0.7 and *dl* X3.1 are viable mutant viruses containing large internal deletions in both copies of the IE-1 gene (Stow & Stow, 1986; Sacks & Schaffer, 1987). Despite retaining their ability to replicate in a range of cell lines, these mutant viruses show a 10-100 fold reduction in virus yield compared to the wild type virus (Stow & Stow, 1986; Sacks & Schaffer, 1987). *dl*1403 contains a 2126bp deletion removing the majority of the Vmw110 coding sequences and approximately 750bp from the 3' end of LAT (Stow & Stow, 1986). *dl*1403, like *in*1814 (Ace *et al.*, 1989), has a high particle:pfu ratio on BHK cells, which means at a low MOI the abolition of Vmw110 function results in a deficiency in the initiation of plaque formation. Analysis of polypeptide profiles demonstrated that the function of Vmw110 is dispensable at a high MOI (Stow & Stow, 1986). The phenotypes of the viral mutants FXE (lacking the zinc finger region) and D22 (containing a 26 amino acid deletion in region 1) were very similar to that of *dl*1403 (Everett, 1989). These results correlated with those obtained in the transfection assays, where the FXE and D22 deletions had an adverse effect on the activity of Vmw110 alone and in the presence of Vmw175 (Everett, 1987b, 1988).

1.2.5.3 Vmw63

UL54 encodes Vmw63, a phosphorylated protein which is found in the nucleus of infected cells (Honess & Roizman, 1974; Wilcox *et al.*, 1980; Ackerman *et al.*, 1984; Knipe *et al.*, 1987).

A pivotal role for Vmw63 in viral gene expression was originally implicated by work carried out using viruses containing temperature sensitive lesions or deletions in the IE-2 gene (Sacks *et al.*, 1985; McCarthy *et*

al., 1989, Smith *et al.*, 1991). Northern blot analysis demonstrated that a ts Vmw63 mutant showed a decreased level of expression of many γ_1 genes and failed to induce the expression of γ_2 genes at the NPT. These defects in late gene expression cannot be attributed to abnormal viral DNA replication (Sacks *et al.*, 1985; Sandri-Goldin *et al.*, 1981). In contrast, some immediate early and early gene products were overproduced relative to wild type HSV-1 infected cells, suggesting an essential role for Vmw63 in the modulation of early and late gene expression. (McCarthy *et al.*, 1989; Sacks *et al.*, 1985). Pulse-labelling experiments also detected overexpression of Vmw175, Vmw110 and Vmw63 in mutant infected cells, suggesting that Vmw63 is involved in the negative regulation of IE gene expression during infection (Rice & Knipe, 1988).

Additional evidence supporting an essential role for Vmw63 in viral gene expression was obtained from transfection experiments. Everett (1984a) showed that plasmid encoded Vmw63 enhanced the expression from a γ_1 gene promoter, that of Vmw155, when Vmw175 and Vmw110 were also present in transient transfection assays. In contrast, in the absence of Vmw175 and Vmw110, Vmw63 alone has very little effect on expression from either HSV or heterologous promoters (DeLuca & Schaffer, 1985; Everett, 1986; Gelman & Silverstein, 1987; Sekulovich *et al.*, 1988). Using a range of target promoters representing all temporal classes, Sekulovich *et al.* (1988) showed that, in the presence of Vmw175 and Vmw110, Vmw63 can function as a transrepressor or transactivator depending on the target promoter. In addition, through the use of transfection assays, Vmw63 was shown to increase CAT activity from a γ_1 promoter, Vmw155, whereas an early promoter, that of TK, was repressed (Sekulovich *et al.*, 1988). These results are consistent with the proposed *in vivo* role of Vmw63 in the switch off of early functions and the activation of late functions (Sacks *et al.*, 1985).

The activation domain has been mapped to the C-terminal half of the polypeptide, encompassing a region of approximately 250 amino acids (Hardwick *et al.*, 1989; Rice *et al.*, 1990; McMahon & Schaffer, 1990) while the repressor activity was mapped to the C-terminal 78 amino acids containing a potential zinc finger domain similar to that reported in the glutocorticoid receptor DNA-binding domain (Berg, 1986, 1988; Green *et al.*, 1988). Vmw63 is retained on a metal chelating column in the presence of zinc (Vaughan *et al.*, 1992) and appears to posses single stranded DNA binding activity (Hay & Hay, 1980; Vaughan *et al.*, 1992). In addition, it is possible that Vmw63 also interacts directly with Vmw175 and Vmw110 as it has little effect on its own in transfection assays, (Rice & Knipe, 1988) and Vmw175 is modified to a

slower migratory form in the presence of Vmw63 (Su & Knipe, 1989). Vmw175 itself does not possess a zinc finger and is therefore not retained on a zinc column. However, in the presence of Vmw63 there is some retention of Vmw175.

Recent analyses of several in-frame deletion mutants has identified important functional regions in the N-terminal half of Vmw63 (Lam *et al.*, Abstract, 17th International Herpesvirus Workshop, 1992). A recombinant virus containing a deletion in the highly acidic region between amino acids 16 to 63 shows a reduced rate of viral DNA synthesis. Another mutant, lacking amino acids 138 to 152, encodes a mutant Vmw63 polypeptide that localizes to the nucleus, but is excluded from the nucleolus. This domain, composed solely of arginine and glycine residues, is similar to a region found in several cellular proteins implicated in nuclear RNA processing. Sequences located between N-terminal 109 and 138 residues appear to contain a nuclear localization signal (Lam *et al.*, Abstract, 17th International Herpesvirus Workshop, 1992).

Recent results suggest that Vmw63 acts, at least in part, at the post-transcriptional level to regulate the expression of IE and L gene products (Smith *et al.*, 1992, Sandri-Goldin & Mendoza, 1992). This work revealed an apparent lack of correlation between the rate of synthesis of new IE and E mRNAs, and their accumulation. In the absence of functional Vmw63, there was an accumulation of IE polypeptides despite a significant decrease in the level of synthesis of new transcripts. In contrast, under the same conditions, late polypeptides failed to accumulate despite the synthesis of new transcripts being unimpaired. Sandri-Goldin and Mendoza (1992) demonstrated in transfection assays, that the activation function of Vmw63 in conjunction with Vmw110 and Vmw175 is not dependent of the promoter, but instead correlates with different mRNA processing signals. Further evidence that Vmw63 functions via a post-translational mechanism was obtained by Chapman *et al.* (1992) who showed that Vmw63 alone can increase expression from a retroviral vector containing Moloney Murine Leukemia virus (MoMuLV) sequences. This activation was dependent on the 3' LTR, and was not affected by the promoter (Chapman *et al.*, 1992). Therefore, it is likely that Vmw63 exerts this effect through a role in processing or stabilization of transcripts (Rice & Knipe, 1990; Smith *et al.*, 1992; Chapman *et al.*, 1992).

Phelan *et al.* (Abstract, 17th International Herpesvirus Workshop, 1992) reported that the expression of functional Vmw63 is required for LPF (late polyadenylation factor) activity, which selectively increases the usage of a virus late poly(A) site in preference to an IE site (McLauchlan *et al.*,

1989). In addition, Sandri-Goldin and Mendoza (1992) demonstrated that Vmw63 reduces the level of spliced target mRNAs in transfection experiments, suggesting that Vmw63 may interfere with host cell splicing. Immunofluorescence studies illustrated that Vmw63 is distributed in a punctate manner and appears to partially co-localize with the U2snRNP (Martin *et al.*, 1987; Phelan *et al.*, Abstract, 17th International Herpesvirus workshop, 1992) and that the distribution of SnRNPs in Vmw63 null mutant-infected cells was indistinguishable from the mock (Hardy *et al.*, Abstract, 17th International Herpesvirus Workshop, 1992). These findings suggest a role for Vmw63 in the disruption of the normal splicing apparatus and is consistent with a role in the polyadenylation and/or splicing processes.

1.2.5.4 Vmw68

The US1 gene encodes the IE polypeptide Vmw68 (McGeoch *et al.*, 1985). The primary transcript contains a single intron in the 5' non-coding region. This 5' regulatory domain also encompasses one of the two ORIS elements, involved in DNA synthesis (Preston & Tannahil, 1984).

On polyacrylamide gels, Vmw68 has an estimated molecular weight of 70,000 (Honess & Roizman, 1974; Post & Roizman, 1981). Vmw68 is phosphorylated and accumulates in the nucleus of infected cells (Wilcox *et al.*, 1980). Vmw68 is not an essential polypeptide as a mutant virus harbouring a 500bp deletion in the IE-4 gene grows efficiently on HEp-2 and Vero cell lines (Post & Roizman, 1981). However on a rodent cell line and resting confluent HEL cells, this mutant displayed a growth dependence on MOI and a decreased plating efficiency. In HEL cells, the production of a late chimeric γ_2-TK protein was significantly reduced (Sears *et al.*, 1985a). This work implied the existence of a host cell factor capable of complementing the function of Vmw68 (Sears *et al.*, 1985a).

1.2.5.5 Vmw12

The role of Vmw12 in the viral life cycle is presently undefined. Unlike the four other immediate early polypeptides, Vmw12 is not phosphorylated and is located in the cytoplasm of the host cell (Marsden *et al.*, 1982). This polypeptide is not essential for virus replication in tissue culture, as deletions within the IE-5 gene have little effect on the growth of HSV (Longnecker & Roizman, 1986; Umene, 1986; Brown & Harland, 1987).

1.2.6 Early gene expression

Early gene expression is dependent upon the synthesis of immediate early polypeptide Vmw175 (Honess & Roizman, 1974; Clements *et al.*, 1977). Initial evaluation of the early TK promoter was achieved by microinjecting a series of 5' and 3' deletion mutants into *Xenopus laevis* oocytes and measuring the resulting mRNA accumulation (McKnight *et al.*, 1981). In this heterologous uninduced system, four *cis*-acting sequence elements were identified (McKnight & Kingsbury, 1982) and subsequently shown to be recognition sites for cellular transcription factors (Jones *et al.*, 1985). The promoter/regulatory sequences of the TK gene comprise a proximal TATA homology, a CCAAT box and two GC-rich Sp1 binding sites (McKnight *et al.*, 1981; McKnight & Kingsbury, 1982; Jones *et al.*, 1985). The distance between these *cis*-elements is important for their function (McKnight, 1982; Eisenberg *et al.*, 1985). These same promoter domains were identified in a transient expression assay in mouse L-cells, indicating that early gene regulation is mediated by general cellular transcription factors. *In vitro* transcription experiments subsequently showed that the components required were present in both mock and HSV-infected cells, indicating that the activation of early gene expression is not solely due to binding of a viral regulatory protein (Jones *et al.*, 1985). Analysis of a series of linker scanning mutations in the TK promoter in the viral genome led to the conclusion that TK promoter recognition is quantitatively similar in basal and regulated environments (Coen *et al.*, 1986).

The regulated expression of the gD promoter was investigated in short-term transfection experiments (Everett, 1983). Deletion analysis revealed that all the signals required for fully regulated transcription lie within 83bp of the mRNA cap site, a region which contains the TATA box and GA rich elements (Everett, 1983).

Recently Imbalzano *et al.* (1990) introduced TK promoter mutations into a Vmw175 deficient viral background, and showed that Sp1 and CCAAT binding protein sites are not required for early gene induction. The relative contributions of these binding sites varies depending upon the presence or absence of Vmw175. It was found that Vmw175 can functionally substitute for the cellular transcription factor Sp1 in the absence of Sp1 binding sites. This hypothesis is feasible if the mechanisms of Vmw175 and Sp1 function involve interactions with common proteins, such as TFIID or the Sp1 co-activator or adaptor molecule (Peterson *et al.*, 1990; Pugh & Tjian, 1990; Imbalzano *et al.*,

1990). This may account for the difference in response to Vmw175 by early genes, which frequently contain Sp1 sites, and late genes, which lack such elements.

1.2.7 Late gene expression

The transcription of late genes is dependent upon functional IE products. Work with temperature-sensitive mutants demonstrated that Vmw175 and Vmw63 are essential for full expression from late promoters (DeLuca *et al.*, 1984; DeLuca & Schaffer, 1985; Sacks *et al.*, 1985; Michael *et al.*, 1988). The UL49 late promoter can be activated in the absence of DNA replication, by co-transfection with IE-gene products (Hall *et al.*, 1982). However, the abundant expression of true late (γ_2) genes requires viral DNA replication as an ORIS negative plasmid lacking a functional origin of replication shows markedly reduced, but detectable, levels of expression from the US11 promoter (Johnson *et al.*, 1986a; Johnson & Everett, 1986a; Godowski & Knipe, 1985, 1986). DNA replication appears to induce a cis-acting modification that renders the template DNA permissive for late gene expression (Mavromara-Nazos & Roizman, 1989).

Johnson & Everett (1986a, 1986b) used a transient assay with a plasmid encoded US11 gene linked to an ORIS to identify the sequence requirements for the expression of late genes. Only 31bp of sequence 5' to the cap site were required for abundant late gene expression (Johnson & Everett, 1986b). The TATA box is the only consensus sequence element required 5' of the mRNA cap site. However, the US11 promoter sequences used in these investigations also included sequences 3' of the initiation site (+37) (Everett & Johnson, 1986b). The relevence of these additional sequences was revealed when Kibler *et al.* (1991) demonstrated that sequences outwith the US11 cap/leader region confer true late regulation on a minimal promoter. An oligonucleotide spanning the US11 TATA box was not sufficient to drive detectable levels of expression, unless the US11 cap/leader region (-11 to +39) was placed downstream, and imposed a strict requirement for DNA replication (Kibler *et al.*, 1991). Mutation of the TATA consensus sequence in the gH promoter largely eliminates reporter gene activity (Steffy & Weir, 1991). However, intact TATA elements are not always sufficient for late promoter activity since linker scanning mutants near the transcriptional start sites of the gH and gC promoters markedly reduced promoter activity, implying that functional initiator elements may be active in HSV late genes (Steffy & Weir, 1991).

It has been suggested that only a subset of TATA elements can function as late promoters since the introduction of the early TK TATA sequences into the gC locus does not direct late gene expression (Homa *et al.*, 1988). There appears to be a correlation between promoter complexity and temporal regulation, because removal of the upstream elements of the early gD promoter and linkage to an ORI_S changes the regulation to that of a true late gene (Johnson & Everett, 1986b). Sequences 3' to the cap site have also been implicated as control elements (Michael *et al.*, 1988; Mavromara-Nazos & Roizman, 1989; Homa *et al.*, 1990). Substitution of the UL49 leader for an early TK leader altered the temporal regulation of the hybrid TK gene, strongly suggesting that the leader sequences impart some of the kinetic class specificity to the late gene family (Mavromara-Nazos & Roizman, 1989). In addition, sequences located in the US11 cap/leader region were shown to impose a strict requirement for viral DNA replication on a minimal TATA box promoter (Kibler *et al.*, 1991). However, the gC cap/leader region does not contain any such regulatory sequences (Homa *et al.*, 1988) implying that not all true late genes are controlled in the same fashion as US11. In transient transfection assays, gC and US11 show different requirements for transcriptional activation. gC can be expressed from an unreplicated template (Shapira *et al.*, 1987) whereas US11 requires replication in *cis* (Johnson & Everett, 1986a).

1.3 Herpes Simplex Virus Latency

1.3.1 Definition of Latency

After infection with HSV at a peripheral site, and the invasion of the sensory nerve endings, the virus ascends by retrograde axonal transport (Ochs, 1974; Kristenssen, 1978) in nerve axons to the nuclei of associated sensory ganglia. At this pivotal stage, either viral replication resulting in neuronal destruction occurs or a latent infection is established. Periodically the latent viral genome may be reactivated, either spontaneously or in response to an external stimulus. It is then transported anterograde in axons, passing from the axon to epithelial cells at or near the primary site of infection where a productive infection occurs.

Using a combination of *in vitro* tissue culture models and animal models, latency may be functionally dissected into a least four sequential stages: (i) In the first instance there may be viral replication in the peripheral tissue. In the absence of viral replication in the ganglia a latent state may then be (ii) established and (iii) maintained. Finally, the latent infection may be (iv) reactivated leading to a productive infection at or near the primary site of inoculation

This section (1.3) discusses the various aspects of latency with particular emphasis on the factors involved in the control of reactivation from the latent state.

1.3.2 Establishment and maintenance of latency

1.3.2.1 Site of latent infection

An overwhelming body of evidence has implicated neuronal tissues as the site of latent HSV. Originally a correlation between trigeminal nerve surgery and the development of cutaneous lesions in humans was noted by Cushing (1905). HSV neurotropism was later also demonstrated in animals studies (Doerr, 1920; Goodpasture & Teague, 1923; Friedenwald, 1923; Marinesco *et al.*, 1973). Following the development of experimental reactivation by explantation (Plummer *et al.*, 1970) and co-cultivation with permissive cells (Stevens & Cook, 1971; Stevens *et al.*, 1972; Knotts *et al.*, 1973; Plummer, 1973; Wohlenberg *et al.*, 1979; Harbour *et al.*, 1981), infectious virus was isolated from sensory ganglia of human cadavars (Bastian *et al.*,1972;

Baringer & Swoveland, 1973; Warren *et al.*, 1977, 1978; Lonsdale *et al.*, 1979) and from experimental animals such as the mouse (Stevens & Cook, 1971), rabbit (Stevens *et al.*, 1972; Nesburn *et al.*, 1972) and guinea pig (Scriba, 1976). It has since been demonstrated that, depending on the site of primary infection, latency can be established in any peripheral nervous tissue, including the autonomic ganglia (Price *et al.*, 1975a; Martin *et al.*, 1977; Warren *et al.*, 1978) and adrenal medulla (Cook & Stevens, 1976). *In situ* hybridization (Cook *et al.*, 1974) and immunofluorescence analyses (McLennan & Darby, 1980; Kennedy *et al.*, 1983) have confirmed that latent virus reactivates from the neurons of ganglia.

Latent DNA has also been isolated from the CNS of rabbits (Plummer *et al.*, 1970), mice (Cook & Stevens, 1976; Knotts *et al.*, 1973; Cabrera *et al.*, 1980) and guinea pigs (Tenser & Hsung, 1977) but not from humans, although DNA homologous to HSV DNA has been detected in human brains (Sequiera *et al.*, 1979; Fraser *et al.*, 1981). The incidence of latency in the CNS is much lower than in the sensory ganglia (Cabrera *et al.*, 1980). However, the inability to detect reactivated virus may either reflect the inefficient reactivation of latent virus by explantation or indicate the presence of defective genomes.

The possibility of latent infections within in non-neuronal tissues cannot be excluded as infectious virus has been recovered by cultivation of corneal discs from mice, rabbits, and man (Shimeld *et al.*, 1982; Abghari & Stulting, 1988) previously infected via this tissue. In addition, both HSV-1 and HSV-2 has been recovered from the sites of primary infection in guinea pigs (Scriba, 1976, 1977; Walz *et al.*, 1977; Donnenberg *et al.*, 1980). Futhermore, the prolonged persistence of both HSV-1 and HSV-2 has been demonstrated in skin and subcutaneous tissue cultures derived from the denervated feet of mice following inoculation of the mouse footpad (Al-Saadi *et al.*, 1983; Subak-Sharpe *et al.*, 1984; Clements & Subak-Sharpe, 1988; Al-Saadi *et al.*, 1988). (Clements & Jamieson, 1989) Prolonged treatment of latently infected mice with acyclovir did not preclude the recovery of HSV from either footpad or ocular tissues following explant and culture, suggesting that the virus isolated was a consequence of a latent infection and not due to a low grade chronic infection (Clements & Subak-Sharpe, 1988; Al-Saadi *et al.*, 1988; Claoue *et al.*, 1990).

1.3.2.2 Structure of the latent genome

Following corneal inoculation of HSV, examination of DNA isolated from latently infected murine ganglia and CNS revealed that the latent DNA was in a different physical state from that observed both during acute

infection and in virions. Rock & Fraser (1983) demonstrated a lack of terminal fragments of HSV DNA in latently infected tissues, Southern hybridization revealed that virion joint fragments were present at an approximately 2:1 molar ratio with respect to the unique sequences of HSV demonstrating that the genome termini are covalently linked (Rock & Fraser, 1985). They proposed that the latent HSV DNA exists in an "endless" state and is not monomeric and linear, extensively rearranged or integrated into the cellular genome via the terminal fragments. The endless configuration of latent HSV DNA was later confirmed in murine and human trigeminal ganglia by Efstathiou *et al.* (1986). Buoyant density gradient centrifugation physically separated viral and cellular DNA indicating that the latent genome probably existed as a circular episome (Mellerick & Fraser, 1987). *In vivo*, the latent genome is associated with nucleosomes in a chromatin structure (Deshmane & Fraser, 1989).

Because DNA methylation is associated with transcriptional inactivity (Ehlrich & Yang., 1981; Yisraeli & Szyf, 1984) it has been suggested as the mechanism whereby the latent HSV genome is retained in an inactive form (Youssoufian *et al.*, 1982; Stephanopoulos *et al.*, 1988). In accordance with this proposition, examination of a latent genome established *in vitro* revealed it to be hypermethylated (Youssoufian *et al.*, 1982). In addition, reactivation from latently infected ganglia *in vitro* is enhanced by treatment with hypomethylating agents such as HMBA and DMSO (Whitby *et al.*, 1987; Stephanopoulos *et al.*, 1988; Bernstein & Knaps, 1988; see section 1.4.4). However, demethylation is unlikely to be the underlying control mechanism during latency as DNA isolated from latently infected mouse CNS is not extensively methylated (Dressler *et al.*, 1987).

Taking into consideration the number of neurons containing LAT^+ (latency associated transcripts, section 1.4.4) in conjunction with the amount of viral DNA present per ganglion, Stevens (1989) estimated that there are approximately 20 copies of viral DNA per latently infected neuron.

1.3.2.3 Role of viral factors in establishment of latency

Despite numerous studies using a range of temperature sensitive, insertion and deletion mutants, no specific viral gene product(s) has been detected which is required for the establishment of latency. Initially, a number of *ts* HSV-1 and HSV-2 mutants were tested for their ability to establish and maintain a latent infection in the nervous system of the mouse,

which has a core temperature of 38.5°C. Mutants were judged to have established latency with the detection of mutant virus after explantation and co-cultivation of the appropriate tissue at temperature permissive for mutant virus growth (Lofgren *et al.*, 1977; McLennan & Darby, 1980; Watson *et al.*, 1980; Al-Saadi *et al.*, 1983).

Russell *et al.* (1987a) showed that mutants *ts*K and *in*1411, lacking a functional Vmw175, established latency in an *in vitro* system (Russell & Preston, 1986). As Vmw175 is an essential polypeptide for productive infection, these results indicate that growth and spread of the input virus is not essential for the establishment of latency.

Studies with the Vmw110 deletion mutant, *dl*1403 (Stow & Stow, 1986) have revealed that Vmw110 is also dispensable for the establishment of latency *in vitro* (Russell *et al.*, 1987a) and *in vivo* both in the mouse eye model (Leib *et al.*, 1989a) and footpad model (Clements & Stow, 1989). Furthermore, other Vmw110 deletion mutants, namely *dl*X3.1 and *dl*X0.7, are also capable of establishing a latent infection in murine ganglia (Leib *et al.*, 1989a). LAT expression is not required for the establishment of a latent infection (Steiner *et al.*, 1990; Leib *et al.*, 1989b).

The controversy shrouding the role of the viral thymidine kinase in the establishment of latency has recently been lifted (Tenser *et al.*, 1989; Leist *et al.*, 1989; Coen *et al.*, 1989). It was originally believed that TK deficient mutants showed a reduction in their capacity to establish latency (Price & Khan, 1981; Tenser *et al.*, 1979, 1981). However, Sears *et al.* (1985b) subsequently reported a lack of correlation between the level of viral TK activity expressed by various mutants and the ability to establish latent infections. This situation was further complicated by the identification of the UL24 gene partially overlapping the TK coding sequences (McGeoch *et al.*, 1988; Jacobson *et al.*, 1989). Mutations within the UL24 coding region have a deleterious effect on viral growth in tissue culture (Jacobson *et al.*, 1989) and it is therefore feasible that the observed phenotypes of the TK$^-$ are attributable to lesion in TK, UL24 or both. A defined mutant virus, which lacks 816bp affecting only the TK ORF, was described by Efstathiou *et al.*, (1989). In the mouse ear model, this mutant replicated with reduced efficiency compared to the wild type virus. Although viral specific DNA was not detected in the neuronal tissues of latently infected mice, superinfection of explanted ganglia by wild type virus led to the recovery of the TK$^-$ mutant (Efstathiou *et al.*, 1989).

Prior to the identification of the LATs (Stevens *et al.*, 1987), it was impossible to differentiate between establishment and reactivation as the

ability of a particular mutant to reactivate was used as a marker for its ability to establish latency. Coen *et al.* (1989) used LAT expression in neuronal nuclei as a marker for latent infection and demonstrated that and ACV-resistant HSV-1 mutant specifically deleted in the TK gene established latency. The TK^- mutant was recovered following superinfection of the mouse trigeminal ganglia with a replication incompetent mutant (Coen *et al.*, 1989). In addition, Kosz-Vnenchak *et al.* (1990) showed, with the detection of nuclear LAT RNA expression, that defined TK^- mutants establish latency in the neurons of mouse trigeminal ganglia after corneal inoculation. Therefore, TK deficient viruses can establish latency efficiently, but are unable to reactivate (Efstathiou *et al.*, 1989; Coen *et al.*, 1989; Kosz-Vnenchak *et al.*, 1990).

Vmw65 is dispensable for the establishment of latency as *in*1814 (Ace *et al.*, 1989) is latency-competent both *in vivo* (Steiner *et al.*, 1990) and *in vitro* (Harris & Preston, 1991). Steiner *et al.* (1990) demonstrated that *in*1814 establishes latency following corneal inoculation, despite the absence of detectable *in*1814 replication at the periphery and trigeminal ganglia. This work also revealed that *in*1814 establishes latency immediately upon reaching the sensory ganglia, between 12-48h p.i. (Steiner *et al.*, 1990). Ecob-Prince *et al.* (Abstract, 17th International Herpesvirus Workshop, 1992) recently demonstrated similar levels of LAT^+ neurons following footpad inoculation of mice with 10^6 particles of either *in*1814 or 1814R. They concluded that *in*1814 establishes latent infections on a particle, rather than pfu, basis in murine dorsal root ganglia. Kristie & Roizman (1987) proposed that the block leading to latency is at the level of immediate early gene expression and therefore the absence of Vmw65 would predispose HSV to latency. Certainly the results of Steiner *et al.* (1990) and Harris & Preston (1991) support the view that the lack of Vmw65 transactivation of the immediate early genes may be the normal route to the establishment of latency. Infection of mice with a recombinant virus expressing Vmw65 under the inducible control of a metallothioneine promoter did not preclude the establishment of latency (Sears *et al.*, 1991). In addition, the neuronal expression of Vmw65 by a transgenic mouse also failed to prevent the establishment of latency (Sears *et al.*, 1991). Therefore, the absence of Vmw65 alone does not account for the establishment of latency, although it may play an auxiliary or pivotal role in the lytic-latent decision.

1.3.2.4 Role of host cell factors in the establishment and maintenance of latency

Given that virtually any HSV deletion mutant can establish latency in mice (Weber *et al.*, 1987; Sears *et al.*, 1985b; Leib *et al.*, 1989b; Katz *et al.*, 1990) and that viral DNA replication (Steiner *et al.*, 1990; Harris & Preston, 1991) and gene expression (Sederati *et al.*, 1989) are not prerequisites for latency, it is possible that cellular factors play an important role in the repression of viral gene expression.

The action of the host immune system may be a determining factor as to the outcome of an HSV infection. Administration of antiviral drugs (Wohlenberg *et al.*, 1976; Klein *et al.*, 1977, 1978a, 1978b, 1979) or prior immunization of animals (Price *et al.*, 1975b; McKendall, 1977; Klein *et al.*, 1978a; Price & Schmitz, 1979; Tullo *et al.*, 1982, 1983) decreases the incidence of latency. During the acute phase of infection, both antiviral IgG (Stevens & Cook, 1974) and interferon (Sokawa *et al.*, 1980) play a role in the clearance of infectious virus from the ganglia.

During establishment of latency *in vitro* at 42°C, the cellular heat shock response may alter the normal pattern of viral gene expression (Russell & Preston, 1986). Such supraoptimal temperatures may induce cell cycle changes which mimic the quiescent nature of neuronal cells or inactivate an essential viral or cellular polypeptide (Russell & Preston, 1986; Harris & Preston, 1991).

Kemp *et al.* (1990) demonstrated that the cellular transcription factor Oct-2, originally identified in mouse neuroblastoma cells, was responsible for the repression of IE gene expression and consequent failure of the lytic cycle in hybrid dorsal root ganglion/neuroblastoma cells. This repressor lacks the activation domain found in the B-lymphocyte form of Oct-2, due to an alternative splicing event. Treatment of hybrid dorsal root ganglion/neuroblastoma cells *in vitro* with NGF increases the level of this neuronal-specific repressor, suggesting that agents which modulate latency may do so by affecting repressor levels (Lillycrop *et al.*, 1992).

The maintenance of the latent state of HSV is dependent upon the presence of nerve growth factor (NGF). Deprivation of NGF leads to the destabilization of latency in primary sympathetic and sensory neurons in culture (Wilcox & Johnson, 1988; Wilcox *et al.*, 1990). In addition *in vivo*, the severing of sensory neurons prevents the transport of NGF to the neural cell

body resulting in reactivation (Carton & Kilbourne, 1952; Walz *et al.*, 1974; Thoenen & Barde, 1980).

1.3.2.5 Latency associated transcripts (LATs)

Transcription of the HSV genome during the latent phase is restricted to the latency associated transcripts (LATs), which were originally identified in murine sensory ganglia by *in situ* hybridization (Stevens *et al.*, 1987; Deatly *et al.*, 1987). Related transcripts have also been detected in latently infected sensory neurons of rabbits (Rock *et al.*, 1988; Wagner *et al.*, 1988) and humans (Croen *et al.*, 1988; Gordon *et al.*, 1988; Steiner *et al.*, 1988; Stevens *et al.*, 1988; Wechsler *et al.*, 1988).

LATs initiate 3' to the IE-1 gene and are partially complementary to the Vmw110 transcripts. There are at least two LAT species, the predominant transcript being approximately 1.8-2.2kb in length, and the less abundant LAT approximately 1.2-1.5kb. The size of the LATs depends upon the strain of the virus and the species of the host animal (Wagner *et al.*, 1988). The predominant 2.2kb LAT species has been detected during lytic infection of tissue culture cells and acute infection of peripheral tissue, albeit with a different subcellular distribution and at an extremely low level in comparison with that found during the latent state (Wagner *et al.*, 1988; Spivack & Fraser, 1987).

Sequence analysis of the LATs revealed that the predominant LAT species contains two putative ORFs, ORF1 and ORF2, which have a potential coding capacity of 36kD and 12kD respectively. ORF2 is retained by the smaller less abundant transcript after a putative splicing event (Wechsler *et al.*, 1988). Despite the presence of an ORF, it is unlikely that the LATs encode a functional protein product due to their nuclear localisation and lack of detectable 3' polyadenylation (Spivack & Fraser, 1987; Wagner *et al.*, 1988). In addition, using serum raised against synthetic polypeptides representing predicted LAT protein sequences Wagner *et al.* (1988) failed to detect any protein products. Lynas *et al.* (1989) sequenced the PCR product obtained by amplification of LAT sequences from total cell RNA extracted from latently infected murine trigeminal and cervical ganglia. The virus strain used was shown to contain a number of base changes and deletions with respect to the published sequences for HSV-1 strains KOS(M) and 17^{+}, which introduced termination signals into the putative ORF2.

The LAT promoter maps to approximately 700bp upstream of the nominal start site of the major LAT and contains a TATA box, potential CCAAT

box and several Sp1 binding sites (Wagner *et al.*, 1988; Wechsler *et al.*, 1989). Other potential regulatory elements in this control region include a Vmw175 binding site (Batchelor & O'Hare, 1990), a cAMP responsive element (Leib *et al.*, 1991) and a palindromic sequence responsible for the binding of the host factor LPBF (Zwagastra *et al.*, 1991), indicating that the control of LAT expression is probably subject to both host and viral factors.

Sequences between -798 to -658 (Batchelor & O'Hare, 1990) and -819 to -662 (Zwaagstra *et al.*, 1990, 1991) upstream of the proposed site of LAT initiation, confer promoter activity when fused to the CAT coding region, in both neuronal and non-neuronal cells, suggesting that the LATs are indeed transcribed from this promoter. The subsequent deletion of this promoter region generates a mutant virus which fails to express LATs (Javier *et al.*, 1988; Leib *et al.*, 1989; Steiner *et al.*, 1989) supporting this hypothesis. The observation of Dobson *et al.* (1989) that the insertion of a β-globin coding sequence downstream of the putative LAT promoter results in β-globin transcripts, but not LATs, in latently infected ganglia provides further evidence that LATs are indeed transcribed from this promoter. The LAT promoter is thought to confer the neuronal specificity of LAT expression (Batchelor & O'Hare, 1990; Zwaagstra *et al.*, 1990, 1991)

Several groups have reported the existence of an 8.3kb or 8.5kb polyadenylated transcript (Dobson *et al.*, 1989; Zwaagstra *et al.*, 1990; Mitchell *et al.*, 1990), whose transcriptional start site was mapped to 24bp downstream of the putative LAT TATA box (Dobson *et al.*, 1989), in both latent and lytically infected cells. The observations that HSV-1 and HSV-2 LAT sequences showed both insignificant conservation and atypical codon usage (McGeoch *et al.*, 1991) and that the 5' terminus of LAT mapped to a splice donor site with a splice acceptor consensus located 1.95kbp downstream, lead to the proposition that the LATs were infect stable introns. Evidence supporting this hypothesis was provided by Farrell *et al.* (1990) who demonstrated that the LAT gene was processed as an intron. Northern blot analysis revealed that the putative LAT intron, cloned into the β-galactosidase gene, was spliced from the primary transcript generating an intact, correctly sized β-galactosidase transcript and the 2.0kb LAT.

However, not all the data are consistent with the hypothesis that the LAT is an intron. If a large 8-9kb primary transcript does exist, then splicing to remove the 2.0kb LAT intron would generate a 6-7kb LAT RNA, which has not been detected. In addition, if the 2.0kb LAT is indeed an intron, then it is an extremely unusual one as it too contains introns. Spivack *et al.* (1991) determined the structure of the 2.0kb and 1.5kb LATs by sequence analysis of

* Doerig *et al.*, (1991), in an in vitro latency model, detected an 80kDal protein from a region covering part of open reading frame 1 (ORF) of the 2.0kb LAT and downstream sequences. However, this is not consistent with the ORF analyses (Weschler *et al.*, 1989; McGeoch *et al.*, 1991; Spivak *et al.*, 1991).

cDNA libraries and RNA PCR products. They showed that the 3' ends of the 2.0kb LAT *in vivo* is upstream of a consensus splice acceptor site. Furthermore, the nucleotide sequences at the 5' and 3' ends of the LAT intron are similar to that seen in eukaryotic protein encoding genes, with the exception of a GC instead of the consensus GT at the 5' end of the LAT intron. Spivack *et al.* (1991) proposed that the GC splice donor site confers tissue specificity on LAT splicing. Removal of the LAT intron brings a 276 nucleotide leader sequence close to ORF1 and ORF2 and relieves translation inhibition of the LAT ORFs by that intron. The data of Spivack *et al.* (1991) is consistent with the existence of a short leader sequence or multiple LAT transcription start sites.

It appears unlikely that LATs function via an antisense modulation of Vmw110 expression, as the disruption of LATs affects neither lytic growth in tissue culture nor acute infection *in vivo*. As previously discussed, LATs are unlikely to act through a protein product (Puga & Notkins, 1987; Wagner *et al.*, 1988; Dobson *et al.*, 1989), although it is feasible that if LATs are in fact stable introns, then part of the 8.3kb or 8.5kb transcript may be protein coding.* As LAT deficient viruses are capable of establishing, maintaining and reactivating from latency, it appears that LATs are not essential at any stage of the lytic cycle (Javier *et al.*, 1988; Sedarati *et al.*, 1989). The possible role of LATs in reactivation is discussed in section 1.3.3.3.

1.3.3 Reactivation from latency

Wildy *et al.*, (1982) defined reactivation as the "reawakening of virus from the latent state, either spontaneously or as a result of external stimuli, so that infectious virus may be isolated."

1.3.3.1 *In vivo* models for studying reactivation

A large number of induction procedures have been developed which reactivate latent HSV *in vivo*. However, most of these treatments bear very little resemblance to the normal physiological stimuli. For example, trauma to the ganglia or neurectomy prevents, or substantially reduces, the transport of NGF to the neural cell body, and leads to the reactivation of latent virus (Walz *et al.*, 1974; Price & Schmitz, 1978; Beyer *et al.*, 1990). Reactivation can also be induced by physical or chemical traumas such as UV irradiation (Blyth *et al.*, 1976), stress, stripping of cellophane tape from the skin (Harbour *et al.*, 1983) and administration of DMSO to the site of peripheral

infection (Harbour *et al.*, 1983). In addition, manipulation of the host immune system by the administration of immunosuppressive drugs (Openshaw *et al.*, 1979; Hurd & Robinson, 1977), superinfection by bacteria (Stevens *et al.*, 1975) and anaphylactic shock (Good & Campbell, 1948) all induce the reactivation of latent HSV.

The rabbit ocular model is a favoured system for studying reactivation from latency *in vivo*, because of the frequent occurrence of spontaneous reactivation (Berman & Hill, 1985; Nesburn *et al.*, 1967) and the availability of a number of methods which reproducibly induce viral reactivation or shedding. These methods include the iontophoresis of epinephrine or 6-hydroxydopamine into the corneas (Hill *et al.*, 1985; Shimomura *et al.*, 1983), systemic or topical treatment with steroids (Spurney & Rosenthal, 1972), and the intrastromal injection of water (Gordon *et al.*, 1990). In contrast, spontaneous reactivation is not seen in mice, and although guinea pigs with an apparent latent HSV-2 infection display frequent spontaneous recurrences, it is possible that these lesions represent a persistent infection (Scriba *et al.*, 1976; Stanberry *et al.*, 1982, 1985). Guinea-pigs are also known not to react to induction procedures used in other systems (Roizman & Sears, 1987).

However, *in vivo* only a very small percentage of neurons in the sensory ganglia harbour latent infections, thus making investigations both difficult and expensive. The heterogeneity of the neural tissues and complexity of the host environment complicates the interpretation of these results (Ho, 1992).

1.3.3.2 *In vitro* systems for studying reactivation

The reactivation of latent HSV was originally assayed by the explantation and *in vitro* culture of latently infected ganglia (Stevens & Cook, 1971). The sensitivity of co-cultivation is greatly enhanced by prior disaggregation of ganglia with trypsin/EDTA treatment (Nesburn *et al.*, 1980). However, while these explant techniques provide an assay for the presence of latent virus, they may not accurately reflect events in the host environment.

A number of tissue culture systems have been developed in which a uniform population of cells may be latently infected and subsequently reactivated by a number of treatments, many of which bear little resemblance to the normal physiological stimuli. Reactivation in tissue culture systems may be facilitated by temperature downshift from 40°C to 37°C (Wighdahl *et al.*, 1981) or the withdrawal of inhibitors of DNA

replication (Cook & Brown, 1987). Wilcox and Johnson, (1987, 1988) have developed an *in vitro* latency system using primary rat sympathetic neurons which may be comparable to the *in vivo* situation as they showed that the addition of anti-NGF antibodies induced reactivation.

An *in vitro* latency system for HSV-2 has been described by Russell and Preston (1986) which does not employ inhibitors of viral DNA replication. Latency is established at a low m.o.i. in HFL cells at the supraoptimal temperature of 42°C, and a stable latent state is maintained at 37°C, a temperature permissive for virus growth. Latent HSV-2 may be routinely reactivated by superinfection with *ts*K at the NPT (38.5°C) or with HCMV. Preston and Russell (1991) have subsequently demonstrated that the latent HSV-2 genome is predominantly non-linear in the *in vitro* system, which is consistent with the *in vivo* situation (Section 1.3.2.2)

1.3.3.3 Role of viral factors in reactivation

The identification of viral factors involved in reactivation began with the demonstration that latent HSV in tissue culture cells could be reactivated by superinfection with *ts* mutants of HSV or HCMV (Colberg-Poley *et al.*, 1979; Wigdahl *et al.*, 1983; Lewis *et al.*, 1984). Given that both *ts*K and *in*1411, which lack functional Vmw175, reactivated latent HSV-2 in a tissue culture system it was concluded that Vmw175 is not required for reactivation *in vitro* (Russell *et al.*, 1987a). Superinfection with *ts*K was as efficient as using a mutant deficient in late viral functions, *ts*I, suggesting that one or more of the IE polypeptides, excluding Vmw175, was responsible for reactivation (Russell & Preston, 1986). The virion components were ruled out as UV-irradiated *ts*K failed to reactivate latent HSV-2 (Russell & Preston, 1986). The Vmw110 deletion mutant, *dl*1403 (Stow & Stow, 1986) failed to reactivate latent HSV-2, suggesting a role for Vmw110 in reactivation (Russell *et al.*, 1987a). This was confirmed by both Harris *et al.* (1989) and Zhu *et al.* (1990) who demonstrated that an Ad5 recombinant virus expressing Vmw110 was capable of reactivating latent HSV-2 *in vitro*. In this system, the D14 mutant harbouring a 41 amino acid deletion from the C-terminal region of Vmw110 (Everett, 1988, 1989) was capable of inducing reactivation, while another Vmw110 deletion mutant FXE (Everett, 1988, 1989) failed to induce reactivation (Harris *et al.*, 1989). FXE lacks 45 amino acids spanning the potential zinc finger domain essential for the transactivation of gene expression by Vmw110 in transient expression assays (Everett, 1984; Gelman & Silverstein, 1985; O'Hare & Hayward, 1985) indicating that the events

leading to reactivation are initiated by the activation of gene expression (Harris *et al.*, 1989).

In vivo, Vmw110 deletion mutants apparently vary in their ability to establish and reactivate from latency (Leib *et al.*, 1989). Vmw110 deletion mutants *dl*X3.1 and *dl*X0.7 are greatly impaired in their ability to reactivate by explantation compared to the wild type virus (Leib *et al.*, 1989), while *dl*1403 is capable of reactivation, although with a reduced efficiency than the wild type virus (Clements & Stow, 1989) or with a small reduction in efficiency (Leib *et al.*, 1989). These differences may be a consequence of additional mutations in the LATs (Section 1.3.2.5), differences in sizes and locations of the deletions in Vmw110 or reflect differences between strains. TK deficient mutants establish latent infections, but as they cannot replicate in neuronal cells, are not recovered as infectious virus during either acute infection or explant reactivation (Leist *et al.*, 1989; Tenser *et al.*, 1989). The viral transcriptional transactivator, Vmw65, is probably not involved in the initiation events leading to reactivation as it is not expressed during latency and prior IE and L gene expression is required for its expression (Stevens *et al.*, 1987; Spivack & Fraser, 1987; Deatly *et al.*, 1987,1988). Work with *in*1814 (Ace *et al.*, 1989) has shown that Vmw65 is not required for reactivation *in vivo* (Steiner *et al.*, 1990) or *in vitro* (Harris & Preston, 1991).

By explantation, the LAT deficient mutant, 1704, was shown to reactivate with slower kinetics than the wild type virus (Steiner *et al.*, 1989), while another LAT deficient mutant, *dl*LAT1.8, showed reduced reactivation (Leib *et al.*, 1989). In contrast Sedarati *et al.* (1989) demonstrated that yet another LAT deficient mutant, KOS8117, reactivated with similar kinetics to the wild type virus, albeit with delayed lytic-phase transcription. The putative LAT promoter was deleted in the reactivation deficient mutants *dl*LAT1.8 and 1704, but remains intact in the LAT mutants which display wild type reactivation. This observation suggests that regions outwith the major and minor LATs may encode important information.

Hill *et al.* (1990) examined a spontaneous LAT deletion mutant and a recombinant LAT^+ virus for epinephrine induced reactivation in the rabbit ocular model. Rabbits latently infected with the LAT^+ virus showed 18/20 reactivation events, while the LAT^- virus showed only 3/22, after the iontophoresis of epinephrine. However, when the rates of spontaneous reactivation were compared, no differences between LAT^- and LAT^+ viruses were observed. Therefore, LATs are not absolutely required for reactivation, but do appear to have a function during epinephrine-induced reactivation (Hill *et al.*, 1990; Trousdale *et al.*, 1991).

Agents which increase the intracellular levels of cAMP are also known to accelerate the reactivation of latent virus from explanted ganglia (Saiz de la Meza *et al.*, 1989). Physiological stimuli such as fever, stress and trauma in addition to experimental reactivation inducing stimuli such as UV irradiation, epinephrine, prostoglandins and the application of retinoic acid, all increase the intracellular levels of cAMP either directly or indirectly through the action of catecholamines and/or arachidonic acid metabolites (Kimberg *et al.*, 1972; Matsuzawa & Nurenberg, 1975; Rodbell, 1980; Levitzki, 1988; Hu & Gudas, 1990). Indomethacin, a potent inhibitor of prostoglandin synthesis, reversibly inhibits HSV-1 reactivation from ganglia (Blyth *et al.*, 1976; Harbour *et al.*, 1983; Kurane *et al.*, 1984). Leib *et al.* (1991) identified a cAMP responsive element (CRE) at position -696 relative to the 5' end of the major LAT. The CRE was responsive to a variety of modulators of cAMP levels and conferred the high level of basal activity of the LAT promoter in neuronal cells. Addition of cAMP analogues or adenylate cyclase activators accelerated reactivation of wild type KOS, but had little effect on a LAT deletion mutant lacking the CRE, *dl*LAT1.8 (Leib *et al.*, 1991). The work of Leib *et al.* (1991), in conjunction with that of Hill *et al.* (1990) suggested a role for cAMP in the modulation of LAT promoter expression and control of viral reactivation.

The enhanced reactivation of latent HSV by hexamethylene bisacetamide (HMBA) and dimethylsulphoxide (DMSO) is discussed in section 1.4.

1.4 Hexamethylene bisacetamide

Hexamethylene bisacetamide (HMBA) belongs to a class of polar planar compounds, including dimethylsulphoxide (DMSO) (Figure 1.4), which have been shown to have a variety of effects on gene expression in a number of different systems.

Both HMBA and DMSO are potent inducers of terminal differentiation in Friend cells (Friend, 1957). This virus-transformed murine erythroleukemic cell line (MELC), provides a model system for the study of the control of erythroid differentiation (Friend *et al.*, 1971; Reuben *et al.*, 1976). HMBA can also induce terminal cell division in a number of rapidly proliferating tumour cell lines (Reuben *et al.*, 1980; Collins *et al.*, 1980; Rabson *et al.*, 1977; Palfrey *et al.*, 1977; Speers *et al.*, 1979). Consequently, many groups are investigating the potential for HMBA as a chemotherapeutic agent in clinical trials as an anti-cancer therapy (Chun *et al.*, 1986; Egorin *et al.*, 1987; Rowinsky *et al.*, 1986; McGuire *et al.*, 1987; Walsh *et al.*, 1987).

HMBA and DMSO have also been shown to increase the speed and efficiency of reactivation of both HSV-1 and HSV-2 from the latent state in a number of animal latency models (Harbour *et al.*, 1983; Stephanopoulos & Bernstein, 1986; Whitby *et al.*, 1987). The growth of the HSV-1 Vmw65 insertion mutant *in*1814 (Ace *et al.*, 1989) in HFL cells is complemented by the presence of 5mM HMBA in the tissue culture medium (McFarlane *et al.*, 1992).

1.4.1 Chemical induction of erythroid differentiation

MELCs provide suitable models for examining the coordinate modulation of gene expression during terminal erythroid differentiation (Takeshita & Benz, 1985; Tsiftoglou & Robinson, 1988). DMSO and HMBA belong to a group of polar planar compounds which are potent inducers of MELC differentiation (Friend *et al.*, 1971; Reuben *et al.*, 1976), HMBA acting at a concentration range 50 times lower than that of DMSO (Palfrey *et al.*, 1977). Exposures of MELCs to a variety of other agents such as actinomycin D (Terada *et al.*, 1978), hypoxanthine (Gusella & Houseman, 1976), short chain fatty acids (Takahashi *et al.*, 1975), ouabain (Bernstein *et al.*, 1976), inorganic selenium compounds (Ebert & Malnin, 1979) and several inhibitors of cAMP phosphodiesterase (Gazitt, 1978), also induces the expression of a developmental programme similar to that observed during normal terminal erythroid differentiation.

Figure 1.4 Chemical structures

The chemical structures of inducers of MELC differentiation, HMBA and DMSO and the newly synthesized rigid and flexible inducers, compound 8 and suberic acid bisdimethylamine respectively (Breslow *et al.*, 1991) are depicted. The chemical structures of the metabolites of HMBA, namely NADAH, DAH, AcHA and AmHA, are also shown.

The chemical symbols employed in the diagram are listed below:-

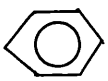
C	carbon
H	hydrogen
N	nitrogen
O	oxygen
S	sulphur
	benzene ring

Figure 1.4 Chemical Structures

HMBA $CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH\text{-}(CH_2)_6\text{-}NH\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_3$

DMSO $CH_3\text{-}\overset{\overset{O}{\|}}{S}\text{-}CH_3$

NADAH $CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH\text{-}(CH_2)_6\text{-}NH_2$

DAH $H_2N\text{-}(CH_2)_6\text{-}NH_2$

AcHA $CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH\text{-}(CH_2)_6\text{-}COOH$

AmHA $H_2N\text{-}(CH_2)_6\text{-}COOH$

Flexible inducer $CH_3\text{-}NH\text{-}\overset{\overset{O}{\|}}{C}\text{-}(CH_2)_6\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH\text{-}CH_3$

Rigid inducer $HO\text{-}HN\text{-}\overset{\overset{O}{\|}}{C}$-(C$_6H_4$)-$\overset{\overset{O}{\|}}{C}\text{-}NH\text{-}OH$

The effects of the five known metabolites of HMBA on differentiation have also been investigated (Reuben *et al.*, 1976, 1978, 1980; Meilhoc *et al.*, 1986; Snyder *et al.*, 1988; Figures 1.4 and 1.5). The deacetylated primary metabolite N-acetyl-1,6-diaminohexane (NADAH), is also an inducer of differentiation in MELCs (Meilhoc *et al.*, 1986), but is a more potent than HMBA in human promyelocytic leukemia cells (HL60) (Snyder *et al.*, 1988). Neither 1,6-diaminohexane (DAH), 6-acetamidohexanoic acid (AcHA) nor 6-aminohexanoic acid (AmHA) are capable of inducing differentiation in MELCs and HL60 cells (Reuben *et al.*, 1976, 1978, 1980; Snyder *et al.*, 1988). However, both AcHA and AmHA can potentiate the activity of HMBA when used in combination (Snyder *et al.*, 1988).

Breslow *et al.* (1991) recently synthesized a series of potent differentiating agents structurally related to HMBA, which appear to fall into two different mechanistic groups. Agents with flexible linear structures such as suberic acid bisdimethylamine (Figure 1.4) are approximately a hundred times more potent than HMBA, and are active differentiators of MELCs, HL-60 and HT-29 cells. In contrast, certain rigid bishydroxamic acids, containing benzene ring spacers (Figure 1.4), are even more active towards MELCs but show poor activity towards HL-60 cells. The rigid molecules display different geometric requirements as they do not act at the same site as HMBA and are not additive with HMBA in their effect. These findings support the idea that the rigid compounds have a different site of action from flexible inducers (Breslow *et al.*, 1991).

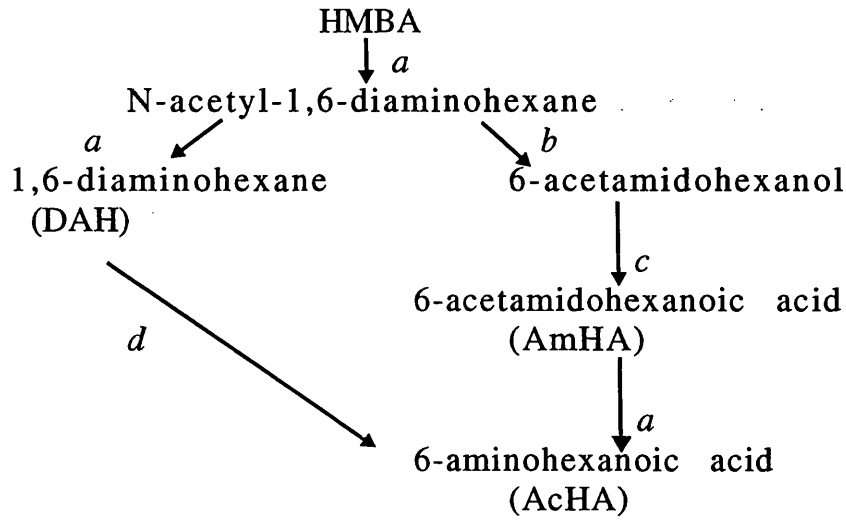
Terminal erythroid differentiation is marked by the accumulation of α- and β-globins and heme-synthetic enzymes, the appearance of erythroid specific membrane antigens and a limited proliferative capacity (Marks & Rifkind, 1978; Tsiftislou & Robinson, 1985). HMBA-induced differentiation of MELCs is a complex multistep process (Chen *et al.*, 1982). In the early "latent" period (within 8-10h), HMBA initiates a number of early metabolic changes which precede irreversible commitment (Chen *et al.*, 1982; Marks *et al.*, 1985; Ramsay *et al.*, 1986). These early events are compatible with cell proliferation and are reversible upon removal of the inducer (Eisen *et al.*, 1978). With more prolonged exposure to HMBA, the MELCs become irreversibly committed to terminal erythroid differentiation. Commitment is defined by the expression of the differentiated phenotype without further exposure to the inducer (Gusella *et al.*, 1976; Fibach *et al.*, 1977; Nudel *et al.*, 1977). Commitment to terminal differentiation is detected as early as 12h post exposure to HMBA, and by 48-60h over 95% of the cell population is committed to differentiation (Nudel *et al.*, 1977). The commitment decision limits the subsequent

Figure 1.5 Pathway of metabolism of HMBA

Proposed scheme for the interconversion between HMBA and its known metabolites. *a*, deacetylase; *b*, monoamine oxidase; *c*, aldehyde dehydrogenase or aldehyde oxidase; *d*, diamine oxidase and aldehyde oxidase.

Adapted from Snyder *et al.* (1988).

Figure 1.5 Pathway of metabolism of HMBA



proliferative capacity of the cell to no more than 5 cell divisions (Gusella *et al.*, 1976; Fibach *et al.*, 1977). The process of commitment appears to require cellular DNA replication in the presence of the inducer (Levy *et al.*, 1975; Friedman & Schildkraults, 1978; Tsiftoglou & Sarterelli, 1981). The kinetics of commitment are consistent with a stochastic or probablistic process which is not identical for all inducing agents (Gusella *et al.*, 1976; Fibach *et al.*, 1977; Figure 1.6).

1.4.2 Alteration of gene expression associated with induction of MELC differentiation

As yet, no genes activiated as a primary response to induction by HMBA or DMSO have been identified, although it has been shown that transcription from a variety of promoters, on plasmids transfected into the cell by electroporation, is stimulated by the addition of inducing agents (Campbell *et al.*, 1990). The induction response requires the continued presence of the inducer and also occurs when *de novo* protein synthesis is inhibited, suggesting that the mechanism involves a change in activity of a factor immediately involved in transcription, probably a post-translational modification. In addition, this effect was not cell-type specific, suggesting that the response may involve a ubiquitous mechanism (Campbell *et al.*, 1990).

In the transient transfection system of Campbell *et al.* (1990) inducibility was shown to be conferred by sequences lying between or including the CCAAT and TATA boxes of the human α- and β-globin genes and upstream from the cap site of the HSV TK gene. However, induction has been detected from rabbit β-globin and SV40 enhancer sequences containing only the TATA box and cap site, suggesting that sequences upstream of the TATA box may not be required for induction in transient transfection assays (Campbell *et al.*, 1990). This view is strengthened by the lack of correlation between upstream sequences and inducibility. Together these observations led to the proposition that HMBA-mediated induction in this system acts at a step intimately involved in transcription initiation.

Figure 1.6 Working model for the effects of HMBA on MELCs

The horizontal arrows represent the proposed sequence of events initiated by HMBA leading to terminal differentiation of MELCs. Modulation of gene expression is indicated as follows:-

(↑) HMBA leads to an increase in expression of this gene

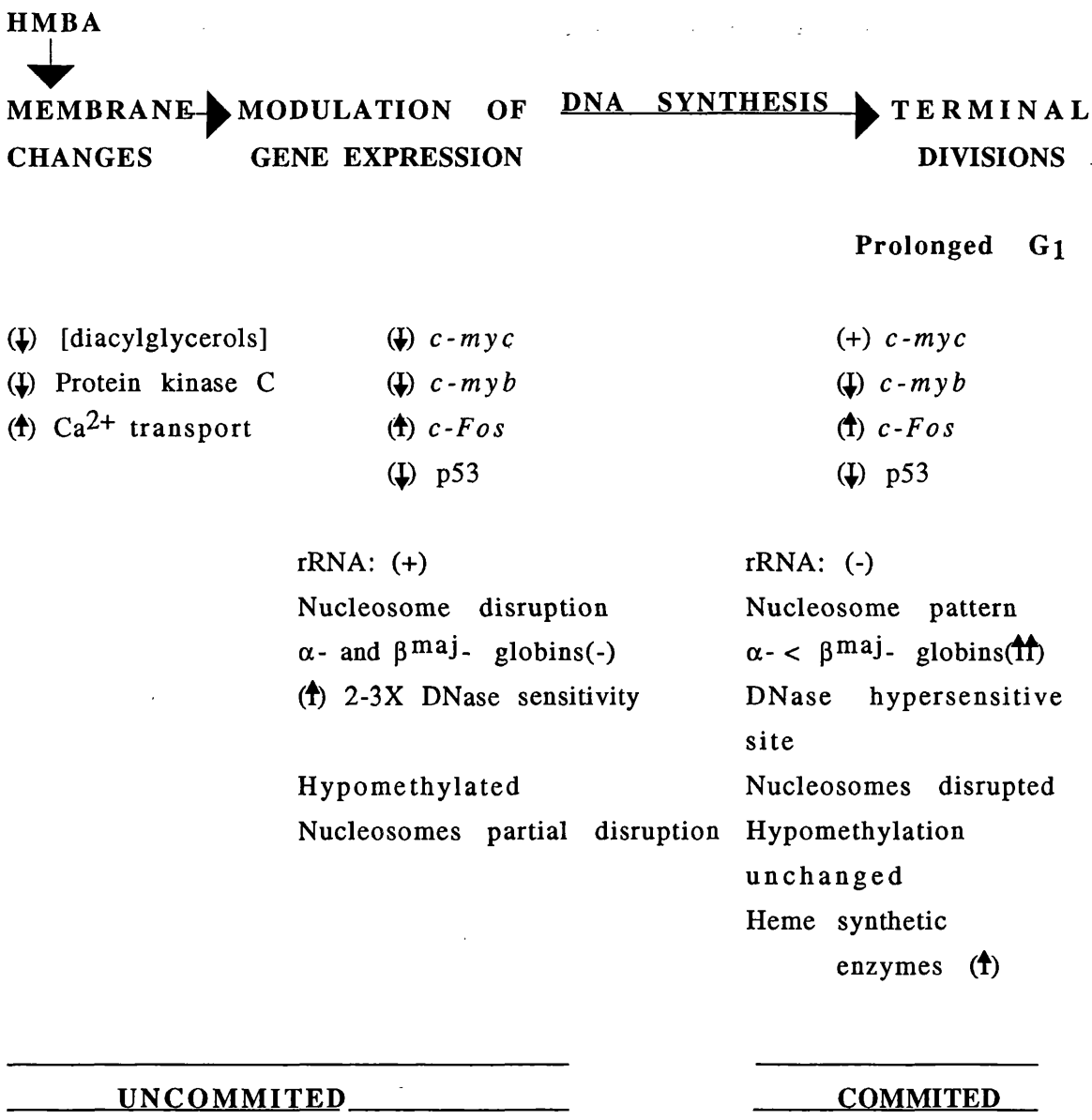
(↓) HMBA leads to a decrease in expression of this gene

(+) gene is actively transcribed in uninduced MELCs or at a level comparable to that in uninduced cells

(-) gene not transcribed or transcribed at a very low level

Adapted from Marks *et al.* (1987).

Figure 1.6 Working model for the effects of HMBA on MELCs



1.4.2.1 Modulation of *c-myc*, *c-myb*, *c-fos* and p53 proto-oncogene expression

Detectable changes in the expression of proto-oncogenes *c-myb*, *c-myc*, *c-fos* and p53 occur within 4 hours of their initial exposure to either 5mM HMBA or 1.5% DMSO (Ramsay *et al.*, 1986; Todokoro & Ikawa, 1986). Although their precise functions are presently unknown, these nuclear proteins have been implicated in the regulation of gene expression related to cell replication (Curran *et al.*, 1984; Klempnauer *et al.*, 1984).

Within 1 hour of exposure to HMBA there is a rapid decrease in *c-myc* mRNA levels and by 4 hours, there is a similar decrease in c-*myb* mRNA, a rise in *c-fos* mRNA, and a transient rise in p53 mRNA levels (Ramsay *et al.*, 1986; Richon *et al.*, 1989). By 12 hours, both the *c-myc* and c-*myb* mRNA levels return to the levels seen in the uninduced cell. *c-myb* mRNA then falls to undetectable levels after 24 hours, whereas the expression of *c-myc* remains at the uninduced level for 48 hours (Ramsay *et al.*, 1986; Richon *et al.*, 1989).

The *c-myb* protein levels correlate with *c-myb* mRNA levels, except at 24h, where despite a decrease in *c-myb* mRNA, the *c-myb* protein level was equivalent to that in the uninduced cell (Richon *et al.*, 1989). The amount of *c-myc* protein correlated with the level of *c-myc* mRNA throughout the entire 48h period of induced differentiation (Richon *et al.*, 1989). In contrast, the levels of p53 mRNA and protein are discordant. After the initial decrease, p53 mRNA levels rise to uninduced levels by 24-28h. However, the amount of p53 protein falls to a very low level by 4-8h, and remains suppressed throughout the remaining period of differentiation (Richon *et al.*, 1989). These differences in p53 mRNA and protein levels can be attributed to changes in the number of mRNAs engaged in translation, and are not due to a decrease in the half life of the p53 protein (Shen *et al.*, 1983; Klochbin *et al.*, 1988).

The early induced changes in *c-myc*, *c-myb*, *c-fos* and p53 mRNA levels are not associated with irreversible terminal differentiation and are not sufficient for commitment (Ramsay *et al.*, 1986). Upon removal of HMBA from the MELC culture medium after 10h exposure, the early changes in *c-myb*, *c-myc*, *c-fos* and p53 have already occurred. However, these cells are not committed to terminal differentiation (Richon *et al.*, 1989). Exposure of MELCs to hemin blocks commitment to differentiation despite the accumulation of globin mRNAs. Hemin has no effect on either the early or

late changes in *c-myc*, *c-myb* or *c-fos* mRNA levels, but still inhibits expression of the full differentiated phenotype (Ramsay *et al.*, 1986). The early decrease in expression of *c-myc* and *c-myb* genes is not blocked by cycloheximide, and may reflect alterations in the state of phosphorylation of the proteins involved in regulating the expression of these proto-oncogenes (Ramsay *et al.*, 1986).

Dexamethasone is an inhibitor of HMBA-induced MELC differentiation which does not block the early suppression of *c-myc* and p53 proteins nor the increase in *c-fos* mRNA. However, dexamethasone does retard the early decrease in *c-myb* mRNA levels and allows it to reaccumulate by 12h to uninduced levels (Ramsay *et al.*, 1986). Therefore, the continued suppression of *c-myb* mRNA appears to be associated with commitment to differentiation (Ramsay *et al.*, 1986). The introduction into MELCs of a plasmid constitutively expressing *c-myb* renders cells unresponsive to DMSO induction of differentiation (Clarke *et al.*, 1988), thus confirming the importance of decreased *c-myb* expression during commitment to differentiation. Constitutive expression of *c-myc* also delays or blocks chemically induced differentiation in MELCs (Copolla & Cole, 1986; Dmitrovsky *et al.*, 1986; Lachman *et al.*, 1986). By exposure to cycloheximide, the transient suppression of protein synthesis in these cells is sufficient to initiate commitment to terminal differentiation. The expression of *c-myc* is associated with continuous proliferation (Marks *et al.*, 1987). However, there are examples of differentiation occurring in the absence of *c-myc* down-regulation (Dotto *et al.*, 1986; Schofield *et al.*, 1987; Sheng-Ong *et al.*, 1987) and reports of a transient decrease in *c-myc* mRNA levels in the absence of differentiation (Blomhoff *et al.*, 1987; Lomo *et al.*, 1987). Darling *et al.* (1989) found that a DMSO-induced decrease in *c-myc* mRNA levels occurred more frequently in tissue culture cells than previously thought. In all the cell lines tested, there is no apparent correlation between the extent and level of transient DMSO-induced decrease in *c-myc* mRNA and the reduction in cell growth. DMSO treatment did not induce a rapid transient decrease in *c-myc* mRNA levels in a human colon carcinoma cell line or in HeLa cells (Darling *et al.*, 1989).

The continued presence of HMBA is required for the suppression of *c-myb* and p53 protein levels. If HMBA is withdrawn, even when more than 90% of the MELCs are irreversibly committed, both proteins reaccumulate (Richon *et al.*, 1989). Therefore, reexpression of *c-myb* and p53 genes does not reverse the commitment process (Richon *et al.*, 1989).

1.4.2.2 Modulation of Protein Kinase C (PKC) activity

Other metabolic changes induced by HMBA during the latent period include alterations in membrane permeability involving Na^+, K^+ and Ca^{2+} flux (Bernstein *et al.*, 1979; Chapman, 1980), changes in cell volume (Gazitt *et al.*, 1978), a transient rise in cAMP levels (Gazitt *et al.*, 1978) and a suppression of rRNA synthesis (Reuben *et al.*, 1980). Rapid changes in phosphatidylinositol turnover are observed after induction, together with a decrease in PKC levels (Faletto *et al.*, 1985; Melloni *et al.*, 1987). Within 2h of exposure to the inducer, there is a significant decrease in the level of the phosphatidylinositol metabolites, inositol-triphosphate and diacylglycerol, which precede the early decrease in *c-myc* (Falleto *et al.*, 1985). Diacylglycerols specifically activate native membrane bound PKC (Kishomoto *et al.*, 1983) and also inhibits MELC differentiation. Interestingly, phorbol esters, such as 12-0-tetradeconyl-phorbol-13-acetate (TPA) and phorbol 12-myristate 13-acetate (PMA) mimic diacylglycerols in that they strongly inhibit MELC differentiation through the activation of PKC and the subsequent reduction in phosphatidylinositol turnover (Faletto *et al.*, 1985). Based on these observations, Faletto *et al.* (1985) suggested that the DMSO-induced reduction in diacylglycerol levels was related to the initiation of differentiation.

Several groups have suggested a role for PKC in chemically induced MELC differentiation (Melloni *et al.*, 1987). Induction by HMBA was shown by Marks *et al.* (1987) to be associated with the appearance of a soluble proteolytically activated form of PKC, PKM, in the cytosol. In addition, Melloni *et al.* (1987) showed that PKM activity is both Ca^{2+} and phospholipid independent. These changes in protein kinase distribution and activity occur within 30-45min of induction (Melloni *et al.*, 1987; Balazovich *et al.*, 1987).

Leupeptin is an inhibitor of calpain, an enzyme responsible for catalysing the conversion of membrane bound native PKC to the soluble activated PKM form (Melloni *et al.*, 1982; Pontremoli *et al.*, 1985). Interestingly, leupeptin also blocks HMBA-induced MELC differentiation (Melloni *et al.*, 1987).

The tumour promoters TPA and PMA are potent inhibitors of HMBA-induced differentiation (Yamaski *et al.*, 1977). Treatment of MELCs with TPA or PMA does not block the early changes in *c-myb* and *c-myc* mRNA levels, but does result in a marked decline in total PKC activity (Fibach *et al.*, 1979; Melloni *et al.*, 1987). Interestingly, after the removal of TPA or PMA, the

restoration of HMBA sensitivity is correlated with the reaccumulation of PKC activity (Melloni *et al.*, 1987). In addition, when MELCs are grown to density arrest, there is a marked decrease in PKC activity, rendering the cells resistant to induction with HMBA (Melloni *et al.*, 1987).

Powell *et al.* (1992) recently separated the MELC PKC isozymes by hydroxylapatite chromotography and identified two species. This work confirmed that the major isozyme peak contains PKCα, whereas the minor peak, previously thought to contain PKCβ (Melloni *et al.*, 1987) actually contains the Ca^{2+}/phospholipid independent PKCε (Powell *et al.*, 1992). Vincristine-resistant MELC variants have been isolated which show increased sensitivity to induction by low concentrations of HMBA and commitment in the absence of any detectable latent period. These rapidly differentiating cells show approximately 25% greater PKCε activity than the parental MELCs (Melloni *et al.*, 1989) and are resistant to the inhibitory effects of both phorbol esters and dexamethasone (Melloni *et al.*, 1988). In contrast, HMBA-resistance is associated with a relatively low PKCε activity (Melloni *et al.*, 1989). Furthermore, the introduction of purified PKCε, but not PKCα, into permeabilized MELCs increases the rate of HMBA-induced differentiation (Melloni *et al.*, 1990). Therefore, it appears that PKCε has a important role in the control of differentiation (Melloni *et al.*, 1988, 1989, 1990).

Several groups have proposed that an increase in responsiveness to HMBA may be due to the constitutive expression of a cellular factor(s) that is critical and rate limiting for an early event in the commitment programme (Melloni *et al.*, 1988; Marks & Rifkind, 1989). For example, unlike the parental MELCs, HMBA-induced differentiation in vincristine-resistant variants is not blocked by the inhibition of protein synthesis by cycloheximide (Michaeli *et al.*, 1990) and it is therefore possible that PKCε may either activate or repress a specific target protein through phosphorylation (Richon *et al.*, 1991). Interestingly, PKCε is capable of phosphorylating histones *in vitro* (Powell *et al.*, 1992).

1.4.2.3 Modulation of globin gene expression

The ultimate consequence of HMBA- or DMSO-induced MELC differentiation is a selective and dramatic increase in the transcription of α_1- and β^{maj}-globin genes. The concentration of globin mRNAs steadily increases during HMBA-induced MELC differentiation to levels approaching 100 fold greater than those of uninduced cells (Ross *et al.*, 1972). The α_1- and β^{maj}-

globin mRNAs do not accumulate simultaneously. The α_1-globin is detected by 12-24h (Sheffery *et al.*, 1984) and is in 4 fold excess over β^{maj}-globin by 31h (Orkin *et al.*, 1975). In contrast, β^{maj}-globin transcription is not detected until 24-36h and reaches levels equivalent to α_1-globin by 96h (Orkin *et al.*, 1975; Salditt-Georgieff *et al.*, 1984).

HMBA induces a number of changes in chromatin structure detectable by 12-24h, which precedes the onset of accelerated transcription of the α_1- and β^{maj}-globin genes. DNaseI-hypersensitive sites appear within 100bp of both α_1- and β^{maj}-globin genes (Sheffery *et al.*, 1982, 1983, 1984; Cohen & Sheffery, 1985; Salditt-Geogieff *et al.*, 1984) and approximately 3000bp upstream of the β^{maj}-globin gene cap site (Yu *et al.*, 1983). The DNaseI-hypersensitive site 5' of the second intron of the β^{maj}-globin gene disappears upon exposure to HMBA. Nucleosome disruption is detected in the transcribed regions of α_1- and β^{maj}-globin genes domains. The importance of these changes in chromatin configuration during induced differentiation was highlighted by Sheffery *et al.* (1983). They demonstrated that a variant cell line which displayed some but not all of the HMBA-induced changes in chromatin structure, was resistant to HMBA-induced terminal cell division.

1.4.2.4 HMBA-induced hypomethylation of MELC genome

Exposure of MELCs to HMBA or DMSO results in a transient genome-wide demethylation, commencing at 3h after the addition of the inducer and reaching a maximum level at 6h (Razin *et al.*, 1986, 1988). During this period more than 60% of the 5-methylcytosines are actively replaced by cytosine using enzymatic machinery already present in the uninduced cell (Razin *et al.*, 1988).

Several observations suggest that although hypomethylation is essential, it is not sufficient for differentiation. For example, inhibition of HMBA-induced hypomethylation correlates with a comparable inhibition of differentiation (Razin *et al.*, 1988). In addition, exposure of MELCs to cycloheximide 24h after induction with HMBA completely inhibits differentiation without significantly affecting the state of HMBA-induced hypomethylation (Razin *et al.*, 1988). Hypomethylation is known to precede commitment and coincides with the first appearance of DNaseI-hypersensitive sites in the α_1- and β^{maj}-globin genes (Sheffery *et al.*, 1984; Salditt-Georgieff *et al.*, 1984).

1.4.3 Effect of HMBA on viral replication

Yura *et al.* (1991) investigated the effect of HMBA on the replication of HSV-1 and HSV-2 in HEp-2 epidermal cells, IMR-32 neuronal cells and a range of cells of lymphoid origin. They concluded that the replication of HSV-1 and HSV-2 in epidermal and neuronal cells was markedly enhanced by HMBA at clinical concentrations of 1-2mM and was greater when infection was initiated at a low MOI. In addition, the presence of the antiviral agent acyclovir, did not suppress the HMBA-mediated enhancement of replication *in vitro* in HEp-2 and IMR-32 cells (Yura *et al.*, 1991). However, Bernstein & Kappes (1988) previously concluded that 5mM HMBA had no effect on productive HSV-2 infection in guinea-pig ganglia *in vitro.* These apparently contrasting results may reflect different effects of HMBA that depend upon the MOI. At the high MOI used to infect the ganglia, it is possible that differences in titre between the presence or absence of HMBA are not detected (Yura *et al.*, 1991).

Lymphoblastoid cell lines latently infected with EBV can be induced to produce viral antigens and infectious mature particles upon exposure to a number of agents including TPA, which is an inhibitor of MELC differentiation (Zur Hausen *et al.*, 1978). In addition, exposure to DMSO, or a range of related polar planar compounds, inhibited the TPA-mediated induction of EBV antigen (Bauer, 1983; Yanase & Sigawara, 1989). However, not all agents capable of inducing MELC differentiation are also inhibitors of EBV induction. The non-polar compounds, hypoxanthine and ouabain, capable of inducing MELC differentiation (Marks & Rifkind, 1978), are ineffective inhibitors of TPA-mediated EBV induction (Yanase & Sigawara, 1989). In addition, the more potent the polar planar compound is at inducing MELC differentiation, the more effective it is at inhibiting EBV induction. These observations led Yanase & Sigawara (1989) to propose that the same mechanism by which the polar planar compounds induce MELC differentiation operates for the inhibition of chemical EBV induction.

Exposure of lymphoblastoid cells infected with human immunodeficiency virus (HIV) to DMSO and other polar planar compounds capable of inducing MELC differentiation, prevented the production of infectious HIV. The inhibition of HIV production required the continued presence of DMSO, and pre-exposure of cells to DMSO prior to HIV infection had only a transient inhibitory effect. This led Viza *et al.* (1989) to postulate

that HIV replication is controlled by DMSO indirectly through unstable or transient modifications of host control mechanisms.

1.4.4 HMBA-mediated reactivation of latent HSV

The *in vivo* treatment of neuronal tissues with 5-azacytidine or DMSO enhances the recovery of HSV-1 from latently infected murine ganglia (Harbour *et al.*, 1983; Whitby *et al.*, 1987) and of HSV-2 from latently infected guinea pig neuronal tissues (Stephanopoulos & Bernstein, 1986). Another observation apparently linking hypomethylating agents with enhanced reactivation was made by Harbour *et al.* (1983) who demonstrated that the application of DMSO to mouse skin resulted in the reactivation of HSV in the associated ganglia *in vivo*. Treatment of latently infected neuronal or peripheral tissues with HMBA or DMSO was then shown to enhance the reactivation of both HSV-1 and HSV-2 in animal latency models (Whitby *et al.*, 1987; Bernstein & Kappes, 1988; Leib *et al.*, 1989).

Whitby *et al.* (1987) examined the effects of DNA hypomethylating agents on the reactivation of HSV from latently infected mouse ganglia *in vitro*. The addition of either 200mM DMSO or 8μM 5-azacytidine, both inhibitors of DNA methylation, to the explant medium resulted in the earlier detection of reactivated virus. The presence of 5-azacytidine also increased the incidence of reactivation from either whole or dissected ganglia (Whitby *et al.*, 1987). Bernstein & Kappes (1988) subsequently demonstrated that another hypomethylating agent, HMBA, (Razin *et al.*, 1986) enhanced the reactivation of latent HSV-2 from guinea pig neural and peripheral tissues *in vitro*. HSV-2 was also recovered earlier and from a greater percentage of ganglia treated with 5mM HMBA, than from the untreated control ganglia. After inoculation in the mouse eye and establishment of latency, the Vmw110 deletion mutant *dl*X3.1 could only be reactivated in the presence of DMSO (Leib *et al.*, 1989). The effects of HMBA and DMSO in these systems had previously been attributed to demethylation as the addition of 5-azacytidine or L-ethionine also improved reactivation of latent HSV (Whitby *et al.*, 1987; Stephanopoulos *et al.*, 1988) and because there is a transient decrease in 5-methylcytosine levels during induction of MELC cells (Razin *et al.*, 1988).

The extent of methylation of cytosine residues in the genome has been implicated in the control of mammalian gene expression (Doerfler, 1981; Mohandas *et al.*, 1981), where DNA methylation is correlated with transcriptional inactivity (Ehrlich & Yang, 1981; Yisraeli & Szyf, 1984). This relationship has also been observed in the control of certain viral genes. The

genome of herpes virus saimiri is hypermethylated in non-permissive cells and undermethylated in permissive cell lines (Desrosiers *et al.*, 1979). Treatment of a transformed cell line containing a non-expressed HSV TK gene with the hypomethylating agent 5-azacytidine results in the activation of TK expression (Clough *et al.*, 1982)). Youssoufian *et al.* (1982) examined the state of methylation of the HSV genome in the persistently infected cell line CEM, and found that during non-productive periods the viral DNA was hypermethylated whereas during productive viral replication the viral DNA was not extensively methylated.

Whether the degree of methylation of the latent viral genome determines the occurrence of reactivation or not is a controversial matter. It has not been proved that the effects of DMSO and 5-azacytidine are solely limited to demethylation (Kolata, 1985). However, as latently infected DNA in the mouse CNS is not extensively methylated (Dressler *et al.*, 1987) it is possible that HMBA actually demethylates and induces the expression of a cellular function(s) necessary for reactivation or completion of the lytic cycle (Whitby *et al.*, 1987; Bernstein & Kappes, 1988). McFarlane *et al.* (1992) recently demonstrated that HMBA increases IE gene expression and proposed that a direct stimulation of IE transcription, rather than demethylating activity, accounts for the improvement in reactivation of latent HSV.

1.5 Eukaryotic gene expression

1.5.1 *Cis*-acting DNA control elements

Most known promoters recognised by RNA polymerase II contain an AT-rich region or TATA box approximately 25 to 30 base pairs upstream of the site of transcription initiation (Maniatis *et al.*, 1987). Mutational analyses have revealed that the TATA box is an important determinant for both the accurate positioning of the initiation site and the basal level of promoter activity (Nakajima *et al.*, 1988). Between positions -40 and -100, upstream promoter elements (UPE) such as the CCAAT box and GGGCGG motif have also been identified. UPEs function to increase the basal level of transcriptional activity and both the number and type of UPEs present determine the resulting degree of transcriptional activity (Maniatis *et al.*, 1987). Analyses of promoters have revealed that the level of transcription often progressively decreases as the distance of the UPE from the TATA box is increased. This suggests that some interaction occurs between upstream transcription factors and the basic transcriptional machinery which is favoured when the binding sites are in close proximity (McKnight, 1982; Wirth *et al.*, 1987; Chodosh *et al.*, 1987; Ruden *et al.*, 1988; Lin *et al.*, 1989; Lennard *et al.*, 1989).

DNaseI footprinting (Galas & Schmitz, 1978) and gel retardation assays (Fried & Crothers, 1981) have identified proteins or transcription factors which specifically bind to UPEs. The cellular factor Sp1 binds specifically to GGGCGG sequence motifs found, for example, in the 21 base pair repeats of the SV40 early promoter. As related transcription factors can bind independently to the same motif, a limited number of transcription factors and UPEs can combine in orders and numbers to produce both temporal and gene specific regulation.

Grosschedl and Birnstiel (1980) found that sequences further than 100 base pairs upstream of the histone H2A gene could act in either orientation to restore efficient transcriptional activity to a minimal promoter. Similarly, sequences further than 100 base pairs upstream of the major early cap site of SV40 were shown by deletion analysis to be essential for expression (Bernoist & Chambon, 1981). These sequence elements, denoted enhancers, dramatically increase the rate of transcription from the associated promoter. Enhancers display exceptional positional flexibility as they are able to act from distances greater than 1000 base pairs, are

orientation independent and can function either 5' or 3' to the end of the gene (Moreau *et al.*, 1981; Benerji *et al.*, 1981; Fromm & Berg, 1983).

Enhancers have a modular structure, being composed of a plethora of short sequence motifs (Serfling *et al.*, 1985). Functional dissection of the SV40 enhancer identified three separate domains denoted A, B and C, each of which is in turn composed of multiple subunits (Zenke *et al.*, 1986; Fromental *et al.*, 1988). Domains A and B alone have weak enhancer activity but duplication of either domain separately or in combination increases the strength of the enhancer (Zenke *et al.*, 1986; Herr & Clarke, 1986). This synergistic effect is independent of the relative positions or orientation of domains A and B (Maniatis *et al.*, 1987). The SV40 octamer motif alone does not possess any enhancer activity, but the level of transcriptional activation increases upon duplication of this element and with the number of copies present (Schirm *et al.*, 1987; Ondek *et al.*, 1987). Multiple copies of the glutocorticoid response element (GRE) also result in a similar additive increase in the transcriptional activity (Toohey *et al.*, 1986). A single heat shock UPE does not act over a great distance, although duplication of this motif generates an enhancer-like activity (Bienz & Pelham, 1986). However, the duplication of CCAAT boxes in the far upstream regions of heat shock promoters does not generate enhancer-like activity (Beinz & Pelham, 1986). Therefore enhancer activity increases with the repetition of certain motifs. In addition, a number of sequence motifs present in promoters and enhancers are functionally interchangeable. For example, the octamer motif present in the immunoglobulin enhancer is also seen in a number of promoters (Bohmann *et al.*, 1987).

The activity of different enhancers varies greatly in a condition dependent manner. Certain enhancers, such as the SV40 enhancer, are constitutively active in a wide range of cell types while those of another group, including the immunoglobulin and β-globin enhancers, are both cell type specific and developmentally regulated (Bohmann *et al.*, 1987). Other enhancers enable genes such as *c-fos* (Triesman, 1985) and interferon (Goodbourn *et al.*, 1985) to be induced immediately upon exposure to growth factors and viral infection respectively. Finally, the metallothioneine enhancer represents those which are both constitutively active and inducible (Maniatis *et al.*, 1987).

Like promoters, the different sequence motifs within an enhancer, represent binding sites for cellular factors. Elucidation of the mode of interaction of the transcription factors bound at the UPEs or enhancers with the basic transcriptional machinery at the TATA box would help our

*TFIID is a multisubunit complex containing a small TATA-binding protein (TBP) and other TBP associated factors (TAFs) (Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; Takada *et al.*, 1992)

understanding of the mechanisms involved in transcriptional activation. Maniatis *et al.*, (1987) proposed that there are protein protein interactions between factors bound at the enhancers and promoters, resulting in the looping of the intervening DNA.

1.5.2 Eukaryotic transcription factors

Eukaryotic cells contain three distinct nuclear DNA-dependent RNA polymerases that are responsible for the transcription of different classes of genes (Roeder, 1976). RNA polymerase I catalyses the synthesis of ribosomal RNA precursors within the nucleolus, and represents approximately half the total transcriptional activity within the cell. RNA polymerase II transcribes protein encoding genes, and the elucidation of the mechanisms regulating this activity is central to our understanding of both cellular differentiation and the development of the organism as a whole. As a consequence of this pivotal role in gene expression, most studies have focussed on transcription by RNA polymerase II. RNA polymerase III is responsible for the synthesis of transfer RNA, 5S RNA and snRNAs.

RNA polymerase II alone is unable to initiate accurate transcription *in vitro*. The polymerase associates with a set of general transcription factors that provide both recognition of, and specificity for, minimal promoter sequences (Matsui *et al.*, 1980). TBP binds specifically to the TATA box and prevents nucleosome-mediated repression of promoter activity (Workman & Roeder, 1987). TFIID itself is necessary and sufficient for the commitment of a template to the transcriptional process (Van Dyke *et al.*, 1989; Conway & Conway, 1989). However, when the concentration of TFIID is low, the formation of stable complexes appears to be facilitated by TFIIA (Davison *et al.*, 1983; Reinberg *et al.*, 1987; Samuels & Sharp, 1986).

Following initiation of transcription, a post-initiation complex, containing TFIID and possibly other factors, remains at the promoter (Van Dyke *et al.*, 1988; Hawley & Roeder, 1987; Van Dyke & Sawadago, 1990) suggesting that reinitiations of transcription may proceed by a different mechanism from initiation of transcription. Sawadago & Roeder (1985) and Carcamo *et al.*, (1989) found a regular pattern of decreasing-length transcripts, which was detected after *in vitro* transcription of a guanosine-free template, and proposed to be due to reinitiations. Szentirmay and Sawadago (1991) subsequently identified a reinitiation transcription factor (RTF) essential for efficient reinitiation by HeLa RNA polymerase II *in vitro*.

The basal level of transcriptional activity is greatly enhanced by a class of promoter-specific transcription factors which bind in a sequence-specific manner to UPEs. The binding of transcription factors to UPEs can direct tissue-specific or constitutive expression or confer inducibility on the associated gene. The transcription factor Sp1 specifically binds to GGGCGG motifs present in the upstream regulatory regions of many promoters, including the SV40 early and HSV TK promoters (Jones *et al.*, 1985). *In vitro*, the GGGCGG motif in closest proximity to the promoter confers the strongest stimulation upon the addition of Sp1. For example, although the HSV IE-3 promoter contains five Sp1 binding sites, a truncated promoter retaining only one Sp1 binding site is still stimulated by the addition of Sp1 (Jones & Tjian, 1985). The DNA binding domain of Sp1 contains three zinc finger motifs, which is consistent with its requirement for Zn^{2+} to bind DNA (Jones *et al.*, 1987).

Different trans-acting factors can bind to the same motif. For example there is a family of transcription factors all of which recognise the CCAAT box. The octamer motif, ATGCAAAT, is a target ubiquitous, cell-cycle specific and cell type specific transcription factors. Oct-1 is present in all cell types and has a ubiquitous role in gene activation, whereas Oct-2 expression is restricted to B-cells and neuronal and is responsible for both the tissue-specific expression and activation of immunoglobulin genes. It is Oct-1, rather than Oct-2, that mediates the induction of HSV IE genes (Gerster & Roeder, 1988). Oct-1 is also involved in the cell-cycle regulated expression of human histone H4 and H2B genes (Heintz & Roeder, 1984; Sive & Roeder, 1986; Fletcher *et al.*, 1987).

The nuclear proto-oncogene *c-fos* forms a heterodimer with *c-jun* at regulatory sequences, which is stabilized by a leucine zipper (Chiu *et al.*, 1988).

Protein-protein interactions between regulatory and basal transcription factors are thought to mediate transcriptional activation. This may occur by direct interaction with, or modulation of, the activity of one or more of the basal transcription factors. Alternatively it may function through intermediary proteins known as co-activators or adaptors (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Pugh & Tjian, 1990).

Pugh and Tjian (1990) demonstrated that the addition of Sp1 to a partially purified preparation of RNA polymerase II and general transcription factors, including TFIID, resulted in a five-fold increase in transcription. In contrast, following the replacement of partially purified TFIID with either cloned *Drosophila* or yeast TFIID or a truncated protein

containing only the C-terminal region, the subsequent addition of Sp1 failed to support induced transcription (Pugh & Tjian, 1990). These observations suggest that a component present in the crude fraction of *Drosophila* TFIID facilitated an indirect interaction between Sp1 and the variable N-terminal tail of TFIID in the basal apparatus (Pugh & Tjian, 1990).

It is likely that a series of co-activator molecules exist, each of which connects different transcription factors with the basal apparatus. In support of this hypothesis, Pugh and Tjian (1990) showed that the ability of CTF to support induced transcription via the CCAAT box required a component of the crude human TFIID fraction, which could not however be functionally substituted by the *Drosophila* TFIID fraction containing the Sp1 co-activator. Verification of a role for these co-activators in gene expression requires their purification or cloning.

Several lines of evidence indicate that TFIID provides a target in the basal apparatus for transcriptional activators. Both ATF and the pseudorabies virus IE protein assist the binding of TFIID to DNA (Horikoshi *et al.*, 1988b; Workman *et al.*, 1988). Using affinity chromatography, Stringer *et al.* (1990) demonstrated that Vmw65 has the ability to interact directly with TFIID, and further showed that mutations affecting the activation capacity of Vmw65 also abolish the retention of TFIID. However, these workers did not investigate whether any other transcription factors were also retained (Lin & Green, 1991). The nature of the proposed interaction between an acidic activation domain and TFIID is open to question. Evidence from several groups has suggested that the direct target for an acidic activator is not TFIID, but is an adaptor or co-activator molecule (Pugh & Tjian, 1990; Berger *et al.*, 1990; Kelleher *et al.*, 1990). This molecule would facilitate the interaction between one of the general transcription factors and the activator domain (Lewin, 1990; Ptashne & Gann, 1990). Berger *et al.* (1990) compared the effect of the chimeric activator GAL4-Vmw65, containing the GAL4 DNA binding domain fused to the activation domain of Vmw65, on a basal promoter and on promoters containing the GAL4 upstream activating sequence (denoted GAL4 UAS) or a T-rich upstream activating sequence (denoted dA:dT UAS). Using a crude fraction, transcription from the dA:dT UAS promoter appeared to be activated, being 5 to 10 fold greater than the basal GAL4 UAS level. The addition of GAL4-Vmw65 to a GAL4 UAS promoter resulted in a 100 fold increase in transcriptional activity, but had an inhibitory effect on transcription from a dA:dT UAS promoter (Berger *et al.*, 1990). Mutations which reduce the effectiveness of the acidic activation domain of Vmw65 also showed a reduced inhibitory effect on the dA:dT UAS promoter. As the

addition of oligonucleotides containing GAL4 binding sites restored basal, but not induced, transcription, Berger *et al.* (1990) proposed that a co-activator required for activated transcription is sequestered by GAL4-Vmw65. In agreement with this conclusion, Kelleher *et al.* (1990) demonstrated that GAL4-Vmw65 mediated inhibition of transcription from a dA:dT UAS promoter was relieved upon the addition of a crude yeast nuclear extract, but not by any of the known purified transcription factors such as TFIID. Gill and Ptashne (1988) also showed that the over expression of GAL4 led to a corresponding decrease in expression of genes lacking GAL4 binding sites. This phenomenon, denoted "squelching", was attributed to the titration by GAL4 of another factor with which it interacts (Gill & Ptashne, 1988).

Horikoshi *et al.* (1992), using permuted binding site/gel retardation assays with recombinant yeast and human TFIID, demonstrated that TFIID binding to the Ad MLP induces DNA bending around the TATA element. Based on these observations, they proposed that TFIID-induced bending may either stabilize interactions with other general transcription factors in the pre-initiation complex, or facilitate contacts between general and upstream regulatory factors.

In contrast, Lin and Green (1991) demonstrated, using affinity chromatography, a specific interaction between the acidic activation domain of Vmw65 and TFIIB. This is a highly specific interaction, as substitution of a single amino acid within the acidic domain dramatically reduces both activation (Cress & Triezenberg, 1990; Berger *et al.*, 1990) and TFIIB binding (Lin & Green, 1991). GAL4 (Horikoshi *et al.*, 1988a) and ATF (Horikoshi *et al.*, 1988b) were previously shown quantitatively to alter the DNase I footprint of TFIID on the TATA box. However, it is feasible that these changes are due to an additional factor, as the major footprint is downstream of the initiation site (Horikoshi *et al.*, 1988a, 1988b), in a region normally protected by TFIIB in pre-initiation complexes (Buratowski *et al.*, 1988; Malderaldo *et al.*, 1990).

Taking into consideration all the available evidence (Reinberg *et al.*, 1987; Reinberg & Roeder, 1987a,1987b; Buratowski *et al.*, 1988), Lin and Green (1990) proposed a model to explain how the acidic activator GAL4-AH, containing the DNA binding domain of GAL4 fused to a synthetic amphipathic helix, stimulated transcription *in vitro*. They suggested that following the interaction of TFIID and GAL4-AH with their respective binding sites, GAL4-AH enhances the recruitment of TFIIB to this complex. The remaining transcription factors and RNA polymerase II can then bind to form a stable pre-initiation complex. Therefore, by enhancing the binding of TFIIB, GAL4-

AH indirectly recruits RNA polymerase II to the promoter (Lin & Green, 1991).

A dramatic and synergistic increase in transcription occurs with the strong activator GAL4-Vmw65 upon duplication of GAL4 binding sites (Carey *et al.*, 1990). It was previously suggested that this increase was due to the simultaneous interaction of two GAL4-Vmw65 molecules with a single basal transcriptional component, presumably TFIID (Carey *et al.*, 1990; Lin *et al.*, 1990). However, it is possible that this effect may be due to the association of one of the bound activators with TFIIB and the other with TFIID.

1.5.3 Chromatin structure

The importance of chromatin structure should not be overlooked when considering the control of eukaryotic gene expression. Eukaryotic DNA is packaged into a number of levels of chromatin structure, the basic unit being the nucleosome. Approximately 145 base pairs of chromosomal DNA are wound around a nucleosomal core composed of an octamer of histones, containing two molecules each of H2A, H2B, H3 and H4. Histones are small proteins which contain a high proportion of positively charged residues and thus promote a tight interaction with the negatively charged DNA. This "beads on a string" structure is condensed to form a 100A fibre in which the nucleosomes are loosely packaged. The 100A fibre can undergo further condensation, facilitated by the cooperative binding of non-histone H1, to form a more compact 300A filament (Allan *et al.*, 1982). Histone H1 is bound to inactive chromatin and is considered to be a generalised repressor of gene activity.

Transcriptional activity correlates with an extended chromatin configuration, whereas inactive genes are relatively condensed (Pederson *et al.*, 1986; Yanir & Cereghini, 1986). DNaseI-hypersensitive sites are invariably associated with active genes and are thought to reflect the exclusion of nucleosomes from promoters by sequence-specific transcription factors at *cis*-acting DNA sequences (Elgin, 1988; Gross & Gerard, 1988). Evidence suggests that the interaction of certain transcription factors with *cis*-acting elements may only be required to establish transcriptionally poised chromatin. For example, the immunoglobulin enhancer is required to establish the expression of transfected genes in precursor B-cells but not once lymphocyte maturation has commenced (Wang & Calame, 1987). In addition, DNase I hypersensitive sites associated with the chicken β-globin

domain persist even after the removal of the inducing stimuli and cessation of expression (Weintraub, 1985).

Locus control regions (LCR), previously named dominant control regions (DCR), are *cis*-acting DNA sequences which confer position independent expression upon associated genes (Grosveld *et al.*, 1987; Forester *et al.*, 1987; Evans *et al.*, 1990; Townes & Bohringer, 1990). For example, the LCR associated with the β-globin gene cluster is marked by four nuclease sensitive domains 10 to 20 kbp upstream of the genes (Tuan *et al.*, 1985; Grosveld *et al.*, 1987; Forester *et al.*, 1987). If this LCR is coupled to the promoter and coding sequences of the β-globin gene, then introduced into transgenic mice, globin mRNA is expressed in proportion to the number of gene copies, suggesting that expression is independent of the chromatin environment at the site of integration. In contrast, without the LCR, globin mRNA expression is extremely low and bears little or no relation to the number of integrated genes. Not all human β-globin LCRs function as enhancers in transient assays, despite containing a wealth of transcription factor binding sites. These LCRs only enhance transcriptional activity when integrated into the cellular genome, suggesting that this activity is associated with the alterations in chromatin structure (Tuan *et al.*, 1989; Talbot *et al.*, 1990; Phillipsen *et al.*, 1990; Talbot & Grosveld, 1991). Reitman *et al.* (1990) identified an element within the chicken β-globin gene cluster which confers position independence without high levels of expression, indicating that the stimulatory and position independence effects of LCRs may be separable.

The 200 base pair promoter region immediately 5' of the adult chicken β-globin gene is a tissue-specific DNase I hypersensitive site which, despite being free of nucleosomes, contains a small protected region due to the association of tissue-specific transcription factors. *In vitro*, hypersensitivity of this site is not induced by transcription factors once nucleosomes have formed in the promoter region (Ermersen *et al.*, 1985). Initiation of transcription *in vitro* is also inhibited if eukaryotic promoters are assembled into nucleosomes (Knezetic & Luse, 1986; Losa & Brown, 1987). However, assembly of the preinitiation complex at the promoter prior to nucleosome assembly prevents repression (Gottesfeld & Bloomer, 1982; Bogenhagen *et al.*, 1982; Lorch *et al.*, 1987; Matsui, 1987; Workman & Roeder, 1987; Felts *et al.*, 1990). In fact, a TFIID-enriched nuclear fraction was sufficient to protect the Ad MLP against nucleosome binding (Workman & Roeder, 1987).

Workman *et al.* (1991), using GAL4 derivatives GAL4-Vmw65 and GAL4-AH, demonstrated that the presence of an acidic activating domain alleviated the repression of promoters during nucleosome assembly and facilitated the formation of preinitiation complexes after nucleosome assembly. This effect was shown to be a function of the transactivating domain, as the deletion mutant GAL4(1-94), which contains only the N-terminal DNA binding and dimerization domains (Keegan *et al.*, 1986; Ma & Ptashne, 1987; Carey *et al.*, 1989), does not preclude nucleosome inactivation despite binding to the promoter region (Workman *et al.*, 1991). Based on the available data, Workman *et al.* (1991) proposed that an acidic activation domain stimulates transcription by enhancing the ability of basal transcription factors to compete with nucleosomes for occupancy of the promoter.

Control of the transition from condensed inactive chromatin to a transcriptionally poised template is thought to be modulated by the differential binding of various chromatin proteins (Jackson, 1986; Weintraub, 1988). For example, active chromatin is frequently enriched in HMG 14 and 17 and certain histone variants. Histone H1 is depleted, while post-translational modifications such as histone acetylation is detected. In addition, ubiquitin is known to associate preferentially with H2A in active genes, although the importance of this modification is not fully understood. The degree of methylation of cytosine residues also plays a major role in the control of gene expression (Doerfler, 1981) and such DNA methylation has been associated with the maintenance of the repressed chromatin state (Gross & Garrard, 1987).

2. MATERIALS

2.1 Tissue Culture

2.1.1 Tissue culture media

ETC_{10} - Glasgow modified Eagles medium (GMEM), 10% newborn calf serum, 10% tryptose phosphate broth, 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin.

EHu5 - GMEM, 5% human serum, 10% tryptose phosphate broth, 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin.

EF_{10} - GMEM, 10% foetal calf serum (FCS), 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin.

HeLa medium - Dulbecco's modified Eagles medium (DMEM), 10% FCS, 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin.

293 medium - DMEM, 10% FCS, 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin.

143TK$^-$ medium - DMEM, 10% FCS, 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin, 50µg/ml bromodeoxyuridine (BrdUrd).

HAT medium - DMEM, 10% FCS, 4mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin, 100µM hypoxanthine, 0.4µM aminopterin, 16µM thymidine, 3µM glycine.

Other media routinely used during tissue culture are detailed below:-

Phosphate buffered saline (PBS) A	170mM NaCl; 3.4mM KCl; 1mM Na_2HPO_4; 2mM KH_2PO_4 and buffered to pH7.2.
PBS B	6.8mM $CaCl_2$

PBS C	4.9mM $MgCl_2$
PBS	8 parts PBS A : 1 part PBS B : 1 part PBS C
PBS Tris-saline	140mM NaCl; 10mM KCl; 10mM Tris-HCl; 1mM $MgCl_2$, pH7.0
Trypsin	0.25% (w/v) trypsin (Difco) dissolved in 140mM NaCl; 30mM KCl; 280mM Na_2HPO_4; 1mg/ml glucose; 25mM Tris-HCl (pH7.4); 0.001% (vw/v) phenol red; 100units/ml penicillin; 100mg/ml streptomycin.
Versene	0.6M EDTA in PBS A containing 0.0015% (w/v) phenol red.
Adenovirus agar overlay	Eagles agar overlay medium without phenol red; 0.65% Noble agar; 0.02% FCS; 1.25μM $MgCl_2$.

2.1.2 Tissue culture cells

Baby hamster kidney (BHK) cells

BHK-21 clone 13 cells (Macpherson & Stoker, 1961) were kindly provided by Dr. C.M. Preston. BHK cells were used for the titration and generation of HSV stocks and for calcium phosphate transfections.

Human foetal lung (HFL) cells

Flow 2002 HFL cells were obtained from Flow Laboratories. HFL monolayers were used to study the effect of HMBA on the plaquing of *in*1814.

HeLa cells

HeLa cells were obtained from Flow Laboratories. Whole cell and nuclear extracts were prepared using HeLa cells for *in vitro* transcription and gel retardation analyses respectively.

293 cells

293 cells were derived from a human cell line by transformation by the E1a region of adenovirus type 5 (Graham *et al.*, 1977) and were a kind gift from Dr V. Mautner. 293 cells were used to titrate *dl*312, an Ad5 E1a deletion mutant.

143 TK$^-$ cells

143 thymidine kinase negative human cells were derived from the murine sarcoma virus transformed line R970-5/5 by selection with BrdUrd at 50μg/ml. 143TK$^-$ cells were used to generate TK$^+$ transformed cell lines and were kindly provided by Dr. K. Page.

pTK$^+$ transformed cell lines

The TK$^+$ transformed cell lines, 143TK1, 143TKN and 143TKTaqA were generated from the parental 143TK$^-$ cell line. They were used to study the effect of HMBA on the expression of the HSV-1 TK, HSV-1 IE-3 and the HSV-1 IE-4/5 promoters, respectively, in the cellular genome.

2.2 Virus Stocks

The following viruses were used in this study:- HSV-1 wt strain 17$^+$ and mutant *ts*K (Marsden *et al.*, 1976); UV-irradiated *ts*K (Notarianni & Preston,1982); Vmw65 insertion mutant *in*1814 and rescued 'revertant' 1814R (Ace *et al.*, 1989); *in*1825 contains the Moloney murine leukaemia virus enhancer and promoter (Lang *et al.*, 1983) in place of the Vmw110 promoter in an otherwise wt HSV-1 genome; *in*1820 has both the Moloney murine leukaemia virus enhancer and promoter driving Vmw110 and *in*1814 insertion; *in*1827 has the HCMV enhancer driving Vmw110 in the *in*1814 background; *in*1833 has the SV40 promoter/enhancer driving β-galactosidase; *in*1411 contains an amber insertion in Vmw175, such that no functional Vmw175 is produced. All these viruses were generously provided by Dr. C.M. Preston. Vmw110 deletion mutant, *dl*1403 (Stow & Stow, 1986), was a gift from Dr. N. Stow. Wild type Ad5 and E1a deletion mutant, *dl*312 (Jones & Shenk, 1979), were kindly provided by Dr. V. Mautner.

2.3 Host bacteria

Plasmids were propagated in the *E.coli* strain K12 DH-1 (*rec*A1; *nal*A; r_R-; m_R-; *endo*I$^-$, B$^-$; *rel*A1). Bacterial cultures were routinely grown in suspension at 37°C in L-broth (1% (w/v) NaCl; 1% (w/v) bactopeptone; 0.5% (w/v) yeast extract.

2.4 Plasmids

Clones were grown from seed stocks supplied by Dr. C.M. Preston and Dr. R. Everett.

pA7436- *Sst*I/*Bbv*I fragment of pJR3, containing the Vmw110 regulatory and coding sequences, inserted between *Sst*I and *Sph*I sites of pUC18. (See appendix 1)

pIE3CAT- -331 to +26 of IE3 promoter fused to CAT gene in a vector derived from pBLW2 (Stow, 1986).

pJR3- Fragment containing Vmw110 regulatory and coding sequences inserted into a pUC9 derivative, in which the *Hinc*II site was converted to an *Sst*I site (Everett, 1984a).

pgDCAT- gD promoter linked to CAT coding region and a polyadenylation signal (Everett, 1986).

pLWFU17- Vmw68 coding and regulatory sequences, including upstream TAATGARAT sequence. (See Appendix 1)

pLWP- Vmw68 coding and regulatory sequences, minus upstream TAATGARAT sequence. (See Appendix 1)

pMC1- comprises a 2.7kbp fragment containing the Vmw65 coding sequence in a pUC9 vector (Campbell *et al.*, 1984).

pHCMVIE- HCMV IE promoter (Towne strain). (See Appendix 1)

pML(C_2AT)$_{19}$ - Ad MLP driving G-less cassette (Savadogo *et al.*, 1985). (See Appendix 1)

2.4 Plasmids (cont.)

pRR55- IE promoter and enhancer of HCMV strain AD169 driving CAT (Fickenscher *et al.*, 1989).

pTK1- HSV-1 *Bam*HI p fragment, containing the HSV-1 TK gene, inserted into the corresponding site in the vector pAT153 (Wilkie *et al.*, 1979).

pTKCAT- *Bam*HI-*Bgl*II region of HSV-1 *Bam*HI p fragment containing the TK promoter (+53 to -650), cloned into *Bam*HI site of pBLW2 (Everett, 1986).

pTKN- HSV-1 IE-3 promoter linked to the HSV-1 TK coding sequences, with the non-selected expression of IE-4.

pTKTaqA- HSV-1 IE-4 promoter linked to the HSV-1 TK gene.

2.5 Oligonucleotides

The oligonucleotides used in this study were synthesized by Dr. J. McLauchlan using a Biosearch 8600 DNA synthesizer. The sequences of the top strand of these double stranded oligonucleotides are listed below:-

JD1/JD2 5' AGCTTGCCTCATGAGTGCGGTAATGAGATACGACTG 3'

CC1/CC2 5' AGCTTGCGTCTTGTCATTGGCGAATTCGAACACG 3'

L1/L2 5' AGCTTGGTTTATAGGTGTAGGCCACGTGACCGGGTG 3'

TATA 5' GATCCGGCTAAACTTATACCCCACGCA 3'

027/028 5' AGCTTGCCTCATGAGTATGCTAATGATATTCGACTG 3'

01/01 5' AGCTTGCCTCATGAGTATGCAAATCATGTGCGACTG 3'

2.6 Enzymes

Restriction endonucleases were obtained from Boehinger Mannheim and Bethesda Research Ltd. Boehinger Mannheim also supplied DNA polymerase I (Klenow fragment) and proteinase K. Bovine pancreas RNaseA and lysozyme were purchased from The Sigma Chemical Company Ltd.

2.7 Chemical reagents

The chemical suppliers are as follows:- BDH Chemicals; Beecham Research Laboratories; Bethesda Research Laboratories; Bio-Rad Research Laboratories; Fluka; Kochlight Ltd; May & Baker; Melford Laboratories; Pharmacia Chemicals; Sigma Chemical Company.

2.8 Radiochemicals

All the radiochemicals used in this study were supplied by Amersham International PLC. The specific activities were 450μCi/mmol for [^{14}C]-chloramphenicol; 800 Ci/mmol for [^{35}S]-methionine, 4,000 Ci/mmol for α-[^{32}P]-CTP; 3,000 Ci/mmol for α-[^{32}P]-dNTPs, 40-60 Ci/mmol for [^{3}H]-thymidine.

2.9 Electrophoresis buffers

3X boiling mix (BM):	50mM Tris-HCl (pH6.7); 0.4% SDS; 30% (v/v) glycerol; 5% (v/v) β-mercaptoethanol; 0.1% (w/v) bromophenol blue (BPB).
10X electroelution: (EEB)	5mM sodium acetate; 1mM EDTA; buffer 40mM Tris-HCl (pH7.8).
Gel-elution buffer:	500mM ammonium acetate; 20mM Tris-HCl (pH7.4); 2.5mM EDTA.
Gel buffer: (GB)	1.5M Tris-HCl (pH8.9); 0.4% SDS.

2.9 Electrophoresis buffers (cont.)

Stacking gel buffer: (SGB)	0.5M Tris-HCl (pH6.7); 0.4% SDS.
Tank buffer:	52mM Tris; 53mM glycine; 0.1% SDS.
10X TBE buffer:	90mM Tris; 1mM EDTA; 90mM boric acid (pH8.3).
5X TBE loading:	5X TBE; 1% (w/v) SDS; 50% (v/v) glycerol; 0.1% (w/v) BPB.
Loading Dye: (Promega Kit)	98% (v/v) formamide; 10mM EDTA; 0.1% (w/v) xylene cyanol; 0.1% (w/v) BPB.

2.10 Lysis buffers

Cell lysis buffer:	20mM Tris-HCl; 0.5% SDS; 2.5mM EDTA (CLB) (pH7.5); 0.25mg/ml proteinase K.
Guanidium: thiocyanate (GSCN)	4M GSCN; 50mM Tris-HCl (pH7.5); 0.5% (w/v) N-lauryl sarcosine; 2mM EDTA buffer (pH7.5); 100mM β-mercaptoethanol; 0.3% (v/v) antifoam A.
TK lysis buffer:	20mM Tris-HCl (pH7.5); 2mM $MgCl_2$; 10mM NaCl; 0.5% (v/v) Nonidet P40.

2.11 Nuclear and whole cell extract buffers

Buffer A: 10mM HEPES (pH7.9); 1.5mM $MgCl_2$; 10mM KCl; 0.5mM DTT.

Buffer B: 0.3M HEPES (pH7.9); 1.4M KCl; 30mM DTT.

Buffer C: 20mM HEPES (pH7.9); 25% (v/v) glycerol; 0.42M NaCl; 1.5mM $MgCl_2$; 0.2mM EDTA; 0.5mM phenylmethylsulfonyl flouride (PMSF); 0.5mM DTT.

Buffer D: 20mM HEPES (pH7.9); 20% (v/v) glycerol; 0.1M KCl; 0.2mM EDTA, 0.5mM PMSF; 0.5mM DTT.

Buffer 1: 10mM Tris-HCl (pH7.9); 1mM EDTA; 5mM DTT.

Buffer 2: 50mM Tris-HCl (pH7.9); 10mM $MgCl_2$; 2mM DTT; 25% (w/v) sucrose; 50% (v/v) glycerol.

Buffer 3: 25mM HEPES (pH7.9); 100mM KCl; 12mM $MgCl_2$; 0.5M EDTA; 2mM DTT; 17% (v/v) glycerol.

2.12 *In vitro* transcription solutions

Transcription buffer: 20mM HEPES (pH7.9); 100mM KCl; 0.2mM EDTA; 0.5mM DTT; 20% (v/v) glycerol

Stop solution: 0.3M Tris-HCl (pH7.4); 0.3M sodium acetate; 0.5% SDS; 2mM EDTA; 3µg/ml tRNA

2.13 Southern/Northern transfer and hybridisation solutions

Alkaline transfer: solution 0.4M NaOH; 0.6M NaCl

50X Denhardts: solution 1% (w/v) ficoll; 1% (w/v) bovine serum albumin (BSA) fraction V; 1% (w/v) polyvinylpyrollidone.

2.13 Southern/Northern transfer and hybridisation solutions (cont.)

Neutralising solution:	0.5M Tris-HCl (pH7.0); 1M NaCl
20X SSC:	3M NaCl; 0.3M sodium citrate
Prehybridisation mix:	0.5M sodium phosphate buffer (pH7.0); 0.5% (w/v) SDS.
Hybridisation mix:	0.5M sodium phosphate buffer (pH7.0); 0.5% (w/v) SDS; radiolabelled DNA probe.

2.14 Other solutions

Oligo reaction mix:	250mM Tris-HCl + 25mM $MgCl_2$ buffered to pH8.0, 50mM β-mercaptoethanol; 1M HEPES (pH6.6); 30 OD units/ml pol (N_6).
Destain:	5% (v/v) methanol; 3.5% (v/v) glacial acetic acid.
Fix:	1% (v/v) glacial acetic acid; 9% (v/v) methanol.
Geimsa:	1.5% (v/v) suspension of geimsa stain in glycerol heated to 56°C for 2h and diluted with an equal volume of methanol.
TE:	10mM Tris-HCl; 1mM EDTA (pH7.5).
TNE:	20mM Tris-HCl; 100mM NaCl; 1mM EDTA (pH7.5).

3. METHODS

3.1 Tissue Culture

All cell types were grown as monolayers at 37°C in an atmosphere of 95% air and 5% CO_2, and routinely passaged every 3-4 days. Cells from confluent monolayers were harvested in 20ml medium after the addition of 10ml 1:1 mix of versene:trypsin then 10ml fresh EF_{10}. This cell suspension was then used to seed subsequent monolayers.

HFL cells were propagated in EF_{10} and routinely split 1:4. A confluent HFL monolayer in a 35mm diameter petri dish contained approximately 10^6 cells.

BHK cells were propagated in ETC_{10} and routinely split 1:10. A confluent monolayer in a 35mm diameter petri dish contained approximately 10^6 cells.

HeLa cells were propagated in HeLa medium and routinely split 1:6. A confluent monolayer in a 50mm diameter petri dish contained approximately 3 X 10^6 cells.

293 cells were passaged after using only 10ml versene to remove the cells from the surface of the tissue culture flasks. 293 cells were routinely split 1:6.

The 143TK$^-$ cell line was routinely grown in 143TK$^-$ medium and split 1:10. The pTK$^+$ transformed cell lines derived from the 143TK$^-$ cells were grown in HAT selective medium and routinely split 1:10.

3.2 Herpes simplex virus

3.2.1 Preparation of virus stocks

50-80% confluent BHK cells in roller bottle were inoculated with 2 X 10^5 p.f.u. of appropriate virus seed stock and returned to 37°C for 2-3 days, until c.p.e. had occurred. Roller bottles were shaken vigorously to disperse cells. Cells were pelleted by centrifugation at 3000 revolutions per minute (rpm) for 10min at 4°C in a Sorvall GSA rotor. The supernatant was decanted into fresh centrifuge tubes and provided a source for both cell released virus (CRV) and viral DNA.

3.2.2 Cell associated virus (CAV)

The cell pellet was resuspended in 15ml ETC_{10} and sonicated for two 30sec bursts to release the CAV. The cell debris was pelleted by centrifugation at 2,500 rpm in a Beckman GPR centrifuge for 15min at 4°C. The supernatant, containing the CAV, was dispensed into 1ml samples, frozen on dry ice and stored at -70°C.

3.2.3 Cell released virus (CRV)

CRV was prepared by centrifugation of the original supernatant at 12,000 rpm for 2h at 4°C in a Sorvall GSA rotor. The viral pellet was resuspended in 8ml ETC_{10} and divided into 1ml aliquots and stored at -70°C.

3.2.4 Preparation of viral DNA

The viral pellet obtained after centrifugation of the infected cell supernatant was resuspended in 10ml 10mM Tris-HCl, 1µM EDTA, 0.1M NaCl, 0.1% SDS and 0.25 mg/ml proteinase K. The sample was rotated at 37°C for 16h and two phenol/chloroform extractions, followed by one chloroform only extraction were carried out. Samples were centrifuged at 2,500 rpm for 15min in a Beckman GPR centrifuge to separate aqueous and organic phases. The viral DNA was precipitated by the addition of 2.5 volumes of ethanol and pelleted by centrifugation at 2,500 rpm in a Beckman GPR centrifuge for 10min. The viral DNA was washed in ethanol before lyophilization and resuspension in 400µl TE at 4°C overnight. The viral DNA was stored in aliquots at -20°C.

3.2.5 Titration of HSV stocks

HSV stocks were titrated on 30mm confluent BHK monolayers. The overlying medium was removed by aspiration and 0.2ml of a virus dilution was added to the monolayers. Virus adsorption and penetration was carried out at 37°C for 1h. The plates were rocked gently every 15min, then overlaid with 2ml EHu2 to neutralise unadsorbed virus and prevent the formation of secondary HSV plaques. After 2 days at 37°C, the medium was removed and cells fixed and stained with Geimsa. Visible plaques were counted under the microscope, and the corresponding titre thus determined.

3.3 Adenovirus

3.3.1 Preparation of adenovirus stock

Adenovirus infected HeLa cells were scraped into the overlying medium and transferred to a 15ml Falcon tube. Samples were centrifuged at 2,000 rpm for 10min in a Beckman GPR centrifuge. The supernatant was discarded and the cell pellet resuspended in 500µl PBS Tris-saline. Samples were transferred to a 1.5ml reaction vial and subjected to three freeze thaw cycles (-70° to 37°C). The cell debris was pelleted as above and the supernatant containing the virus stock was transferred to a fresh vial. Adenovirus stocks were stored at -20°C.

3.3.2 Titration of adenovirus stock

Confluent 50mm Petri dishes of HeLa cells were infected with 100µl of the appropriate dilution of adenovirus in PBS Tris-saline. Adsorption was carried out at 37°C for 1h. Cells were overlaid with 5ml medium in the presence or absence of 5mM HMBA, and incubated at 37°C overnight. The following day the medium was replaced with 5ml agar overlay, left to set at room temperature (RT), and returned to 37°C. At days 5,7 and 8 a further 1-2ml of agar overlay was added. At day 10 plaques were ready to be counted. Cells were fixed with 2ml 1:1 gluteraldehyde:PBS overnight at RT. The agar overlay was then "flipped" off and the fixed cells stained with Geimsa.

3.4 Introduction of DNA into tissue culture cells

3.4.1 Calcium phosphate mediated transfection of viral DNA

The method employed was essentially as described by Stow & Wilkie (1976). 30mm plates were seeded with 5 X 10^5 BHK cells, using a haemocytometer to determine the concentration of the cells in the suspension. 0.1µg viral DNA and 1.25µg calf thymus carrier DNA were brought up to 57µl with the addition of distilled water (DW). 66µl 2X HEBS and 9µl 2M $CaCl_2$ were added to form a precipitate. The medium was aspirated from the monolayers and the calcium phosphate/DNA precipitate was gently added dropwise to the cell monolayer. Plates were transferred to 37°C and rocked gently every 15min. After 45min, 2ml medium was added and the cells

returned to 37°C for 3-4h. The medium was removed and 25% v/v DMSO in 1X HEBS was applied at RT for 4min (Stow & Wilkie, 1976). The DMSO was removed and the monolayers washed twice with ETC_{10}. 2ml medium was added and the monolayers were incubated at 37°C for 16h.

3.4.2 Calcium phosphate mediated transfection of plasmid DNA

Plasmid DNA was adjusted to the appropriate quantity of total DNA by the addition of pUC9 to give a volume of 57µl. 66µl 2X HEBS and 9µl $CaCl_2$ were added and the sample vortexed briefly. The medium was aspirated from the plate using a Pasteur pipette, before the gentle dropwise addition of the precipitate. The plates were incubated at 37°C and rocked gently every 15min for 45min, at which time 2ml of "plating medium" (20ml used ETC_{10}; 17.8ml fresh ETC_{10}; 2ml HEBS; 0.25ml 2M $CaCl_2$) was added. A 4min 25% DMSO boost was carried out 3-4 h later as described in 3.4.1.

3.4.3 Lipofection of plasmid DNA

Lipofection Reagent is a 1:1 (w/w) liposome formulation of N-[1-(2,3-dioleyloxy) propyl] -N,N,N -trimethylammonium chloride (DOTMA), a cationic lipid and dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. This reagent spontaneously interacts with DNA forming a lipid-DNA complex resulting in the entrapment of the DNA. Subsequent fusion of the complex with the cell membrane results in efficient uptake and expression of the DNA.

1µg DNA and 10µl Lipofection Reagent were separately diluted to 20µl in DW, then combined in a polystyrene tube and left to stand at RT for 15min. 80-85% confluent HFL cell monolayers were washed twice with Opti-MEM1, a cell culture medium lacking serum. 1ml Opti-MEM1 was then added to the cells, while gently rocking the plates. 40µl of the lipid-DNA complex was added dropwise, as uniformly as possible to the cells. Cells cultures were incubated for 1-5h at 37°C before ending the lipofection with the addition of 1ml EF_{10}. The medium was changed after 24h and the cells harvested at 48h.

3.5 Extraction of cellular and viral nucleic acids

3.5.1 Extraction of whole cell DNA

Cells were scraped into 1ml medium and pelleted by centrifugation at 5,000 rpm in a benchtop microfuge for 15min, before being resuspended in 2ml CLB containing 0.25mg/ml proteinase K, and incubated at 37°C for 3-5h. 150µl 5M NaCl and 2ml phenol/chloroform was added to each sample, vortexed briefly then centrifuged at 5,000 rpm in a benchtop microfuge for 10min. The upper phase was removed and 2.5 vols ethanol and sodium acetate to 0.3M were added. The tubes were inverted a few times resulting in the precipitation of DNA which was pelleted by centrifugation at 13,000 rpm for 10min in a benchtop microfuge. Once dry, the DNA was resuspended in 500µl of TE or DW (Stow *et al.*, 1986).

3.5.2 Separation of nuclear and cytoplasmic DNA

Cells were scraped into 1ml PBS and pelleted by centrifugation at 13,000 rpm in a benchtop microfuge for 2min. The overlying supernatant was discarded and the cell pellet was vortexed before the addition of 0.1ml TK lysis buffer. After another brief vortex, the samples were incubated on ice for 5min, vortexed again, then centrifuged at 13,000 rpm in a benchtop microfuge for 2min. The pellet contained the nuclear DNA and the supernatant contained the cytoplasmic DNA. DNA extractions of both the pellet and the supernatant were carried out as described in 3.5.1.

3.5.3 Extraction of cytoplasmic RNA

The procedure employed for the extraction of RNA from either mock or virus infected cells is essentially as detailed by Ace *et al.* (1989). The protocol described here refers to 60mm diameter Petri dishes of confluent cells. The overlying medium was removed by aspiration and the cells washed with 5ml ice cold PBS. The cells were then scraped into 1ml PBS and centrifuged at 5,000 rpm in a benchtop microfuge for 30sec. The cell pellet was resuspended in 45µl cold 10mM Tris-HCl; 1mM EDTA, and 5µl 5% NP40 was added. The samples were vortexed and incubated on ice for 5min, followed by the addition of a further 5µl 5% NP40. After another brief vortex, the samples were centrifuged at 5,000 rpm in a benchtop microfuge for 2.5min. The

supernatant, containing the RNA, was transferred to a sterile vial containing 30µl 20X SSC and 20µl formaldehyde. Samples were incubated at 60°C for 15min, and stored at 70°C until required. The absorbance at 260nm was measured to determine the concentration of the RNA.

3.5.4 Extraction of whole cell RNA

Whole cell RNA was extracted essentially as described by Chomczynski & Sacchi (1987). 90mm diameter Petri dishes of cells were harvested in 3ml PBS and transferred to a 10ml Sarstedt tube. Cells were pelleted by centrifugation at 2,000 rpm for 15min in the outer ring of a Sorvall SM24 rotor. The supernatant was discarded, and the cell pellet resuspended in 1.8ml GSCN buffer with vortexing. 120µl 2M sodium acetate pH4.0, 1.8ml water saturated phenol and 180µl chloroform/isoamylalcohol (49:1) were added, and the samples vortexed after the addition of each solution. The samples were vortexed for 10sec and incubated on ice for 15min. The phases were separated by centrifugation at 10,000 rpm for 15min at 4°C in a Sorvall SM24 rotor. The aqueous phase was transferred to a fresh tube, leaving behind the interphase because at pH4.0 DNA enters the phenol phase. 1.8ml isopropanol was added and the samples inverted before precipitation at -20°C for 1h. The RNA was pelleted by centrifugation at 10,000 rpm for 15min in a Sorvall SM24 rotor, and resuspended in 300µl GSCN buffer and 300µl isopropanol. The samples were reprecipitated at -20°C for 1h and pelleted as above. Pellets were resuspended in 200µl nuclease free DW, and 20µl 3M sodium acetate and 600µl ethanol was added. After vortexing the samples were left at -20°C at least 16h. The RNA was stored as a precipitate until required. When needed, the RNA was pelleted as above, washed twice with 70% ethanol then dried. Finally the RNA was resuspended in 50µl nuclease free DW and the absorbance determined at both 260nm and 280nm.

3.6 Manipulation of plasmid DNA

3.6.1 Large scale preparation of plasmid DNA

Large scale preparations of plasmid DNA were made using a modification of the protocol by Guerry *et al.* (1973). 5µl of the appropriate glycerol stock of bacteria was inoculated into 10ml L-broth containing 50µg/ml ampicillin and left shaking at 37°C overnight. The overnight culture was added to 200ml L-broth containing ampicillin and shaken at 37°C for

approximately 8h. Chloramphenicol was added to a final concentration of 25μg/ml and incubation continued overnight. The cultures were pelleted by centrifugation at 8,000 rpm for 10min in a Sorvall GSA rotor and resuspended in 5ml 25% sucrose; 50mM Tris-HCl (pH8.0). 2.5ml fresh 5mg/ml lysozyme was added, mixed and left on ice for 30min. 2ml 250mM EDTA pH 7.5-8.0 was added and the samples were incubated on ice for 5min. 1.5ml of both 5M NaCl and 20% SDS were added and the samples thoroughly mixed and left on ice for 2-3h. The cell debris was removed by centrifugation at 20,000 rpm in a Sorvall SS34 rotor for 1h at 4°C. The supernatant was extracted twice with 20ml phenol/chloroform and once with 15ml chloroform. The upper aqueous phase was precipitated by the addition of 2 volumes of ethanol at -20°C for 1h or overnight. Samples were centrifuged at 2,500 rpm for 15min in a Beckman GPR centrifuge, and the resultant soft pellet freeze dried. The pellet was dissolved in 9ml TNE containing 20μl 5mg/ml RNaseA and incubated either at 37°C for 2-4h or 30°C overnight. 0.5ml of 100μg/ml proteinase K solution was added, and the incubation was continued at 37°C for 2-16h. Two phenol/chloroform and one chloroform extractions were carried out as described above. The volume of the final aqueous phase was adjusted to 9ml with DW and 1ml 3M sodium acetate (pH7.0) and 5ml isopropanol were added. After mixing, samples were left to stand at RT for 2h, and centrifuged at 2,500 rpm for 15min at 20°C in a Beckman GPR centrifuge. The supernatant was discarded and the pellet allowed to dry at RT overnight, before being dissolved in 300μl DW. Serial dilutions of the plasmid were run on a mini-gel along side a known standard to quantitate the DNA.

3.6.2 Preparation of "mini-prep" DNA

Colonies for analysis were picked using a sterile toothpick, transferred to a universal containing 2ml L-broth with 50μg/ml ampicillin and placed in an orbital 37°C incubator overnight. 1.5ml was decanted into 1.5ml vial and the bacteria pelleted by centrifugation at 5,000 rpm in a benchtop microfuge for 2min. The medium was removed by aspiration and the pellet resuspended in 100μl STET containing 1mg/ml lysozyme. Samples were then placed in a boiling water bath for 1.25min and centrifuged for 10min at 13,000 rpm in a benchtop microfuge. The supernatant was transferred to a vial containing 400μl 0.3M sodium acetate (pH7.0). 0.5ml isopropanol was added and the samples were incubated at -20°C for 1h. DNA was pelleted by centrifugation at 13,000 rpm in a benchtop microfuge for 5min, dried, before resuspension in 33μl DW. Finally, DNA was analysed

by cleavage with the appropriate restriction enzyme, in the presence of 0.5µg/ml ribonuclease (RNaseA).

3.6.3 Restriction enzyme digests

Restriction enzyme digests were carried out at 37°C for the required time in the appropriate commercial buffer. The number of units of enzymes added was dependant on the activity of the enzyme and the amount of DNA to be cleaved.

3.6.4 Electroelution of DNA from agarose gels

Using a sterile scalpel, the required band was excised from the gel as visualised under long wave UV light. The DNA was isolated by electroelution in 1X EEB at 20mA per sample for 1.5h at 4°C, using equipment designed for this purpose. The DNA was removed in 200µl 1X EEB and transferred to a fresh vial containing 160µl DW; 40µl 3M sodium acetate; 1µl *E.coli* rRNA. A 0.5ml phenol/chloroform and a 0.4ml chloroform extraction were carried out. The DNA was precipitated in an equal volume of isopropanol at RT for 1h. The DNA was pelleted by centrifugation at 13,000 rpm in a benchtop microfuge and the salt removed by a 70% ethanol wash. The pellet was dried and resuspended in the appropriate volume of DW.

3.6.5 Ligation of plasmid DNA

Ligations were performed using 50ng of linearised vector DNA and 200-500ng of purified fragment in 20mM Tris-HCl (pH7.6), 10mM $MgCl_2$, 10mM dithiothreitol (DDT) and 0.6mM ATP and catalysed by 10units T4 DNA ligase. Reactions were incubated at 15°C for 16h.

3.6.6 Preparation of competent bacteria and transfection of plasmid DNA

10ml L-broth was inoculated with 5µl of a glycerol stock of *E.coli* DH-1, and placed in an orbital incubator at 37°C overnight. 2ml of this culture was added to 80ml L-broth and shaken at 37°C until an OD of 0.2 was reached. The bacteria were pelleted by centrifugation at 5,000 rpm for 10 min in a Beckman GPR centrifuge, resuspended in 40ml ice cold 100mM $CaCl_2$, and

placed on ice for 1h. The competent bacteria were pelleted as above and resuspended in 0.8ml ice cold 100mM $CaCl_2$.

2µl ligation mix was added to 100µl competent bacteria and put on ice for 1h. Samples were heat shocked for 1.5min at 42°C and transferred to a universal containing 1ml L-broth and shaken at 37°C for 1-1.5h. 150µl aliquots were spread on agar plates containing 50µg/ml ampicillin and returned to 37°C overnight.

3.6.7 Ampicillin agar plates

L-broth agar was melted then left to cool until hand hot, at which time ampicillin was added to a final concentration of 50µg/ml. The agar was then poured into 90mm sterile Petri dishes and allowed to set. Plates were dried in an inverted position at 37°C for 1h before use and stored at 4°C until required.

3.6.8 Preparation of bacterial glycerol stocks

5ml L-broth was inoculated with a particular bacterial isolate, and placed in an orbital incubator at 37°C overnight. 1ml of the culture was centrifuged at 5,000 rpm in a benchtop microfuge for 1min. The supernatant was discarded and the pellet resuspended in 0.5ml 2% bactone peptone and 0.5ml 80% glycerol. Glycerol stocks were stored at -20°C.

3.7 Electrophoretic separation of nucleic acids

3.7.1 Agarose minigels

Rapid examination of small quantities of DNA was achieved using mini-gels (100mm X 70mm X 7mm) consisting of 30ml 0.8% agarose in 1X TBE. 10µl samples were loaded in 1X TBE loading buffer and electrophoresed at 40mA for approximately 1h. 5µg ethidium bromide was added to the running buffer and the samples were visualized by exposure to UV light and photographed on type 667 Poloroid film.

3.7.2 Large agarose gels

DNA restriction fragments were analysed and prepared using 300ml horizontal 0.8% agarose gels (260mm X 160mm X 7.5mm) containing 0.5µg/ml

ethidium bromide in a 1X TBE running buffer. DNA samples were mixed with 1X TBE loading buffer before electrophoresis at 40mA for 3-4h.

3.7.3 Non-denaturing polyacrylamide gels

Gel retardation assays and the purification of radiolabelled oligonucleotides were carried out using vertical gels (260mm X 160mm X 1mm) and the appropriate concentrations of acrylamide in 0.5X TBE. 50ml of 8% acrylamide was prepared from a stock solution of 20% acrylamide and 1% N-N' methylene bisacrylamide, with 0.5X TBE. Gels were polymerised with the addition of 0.5ml freshly prepared 10% ammonium persulphate and 50µl TEMED. Samples were loaded in 1X TBE loading buffer using a capillary tube. Electrophoresis was carried out at 250V for 1-1.5h, until the bromophenol blue (BPB) band was 2" from the bottom of the gel.

3.7.4 Denaturing urea/polyacrylamide gels

The RNA products from *in vitro* transcription reactions were analysed on denaturing 7M urea 6% polyacrylamide gels. 6% acrylamide was prepared from a stock solution of 20% acrylamide, 7M urea in 1X TBE, using 10M urea to bring the solution to the required volume. Catalysts were added as previously described. (Section 3.7.3) The gel was pre-run in 1X TBE for 10min at 250V. The wells were flushed with 1X TBE buffer and the samples loaded. Electrophoresis was continued at 250V for 3-4h, until the BPB dye just ran off the gel. The gels were dried under vacuum, then subjected to autoradiography.

3.7.5 SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was employed to analyse the viral polypeptides produced after infection of tissue culture cells at both immediate early (IE) and late (L) times. Glass plates were soaked overnight in dilute HCl, then rinsed in DW before use. A 12% acrylamide gel was poured into a prepared gel sandwich (260mm X 160mm X 1mm) and overlaid with 0.25X gel buffer (GB) until polymerised. The 0.25X GB was poured off and the interface washed twice with stacking gel (SG). The SG was added and a teflon well former inserted to form sample wells. Once polymerised, the wells were washed out with tank buffer. Gels were run in tank buffer at 35mA for 3h. Gels were soaked overnight in a

50:50 mix of gel fix:gel stain, and dried under vacuum then subjected to autoradiography.

3.7.6 Denaturing agarose/formaldehyde gels

RNA extracted from TK^+ transformed cell lines under various conditions was prepared for Northern blotting by electrophoresis on denaturing formaldehyde agarose gels (260mm X 160mm X 5mm). The formaldehyde gel was prepared by boiling 3.6g agarose in 261ml nuclease free DW. The agarose was cooled to 55°C then 30ml 10X MOPS and 9ml formaldehyde was added, and the gel was poured immediately. 5µl 10X MOPS and 8.75µl formaldehyde was added to 12µl RNA samples and incubated at 65°C for 15min. 10µl loading dye (0.25% BPB; 0.25% xylene cyanol; 1mM EDTA; 50% glycerol) and 1µl 1mg/ml EtBr was added, and the samples loaded directly into the wells. Electrophoresis was carried out at 70-80V in a fume hood, until the BPB band had travelled 10cm from the wells.

3.8 Analysis of viral protein synthesis

3.8.1 Viral IE-protein synthesis

IE polypeptides were generated at 38.5°C as described by Preston *et al.* (1978). HFL cells were infected with 500 particles of virus per cell, in the presence of 25µg/ml cycloheximide. Plates were transferred to 38.5°C for 3h post-adsorption, before the release of the cycloheximide block by three 1.5min washes with fresh prewarmed culture medium. Polypeptides were radiolabelled with EF10 medium containing 0.3ml of 50µCi/ml [^{35}S]-methionine in the presence of 1µg/ml actinomycin D for 90 min. At the end of the labelling period, the supernatant was removed and the cultures lysed and harvested as described by Marsden *et al.* (1978). Cells were washed with 2ml ice cold PBS, followed by 1ml ice cold DW. 200µl ice cold 10mM Tris-HCl (pH7.5); 5mM EDTA was added and the plates left on ice for 5min. Cells were removed from the plates by squirting the Tris-HCl/EDTA over them, before transfer to a vial containing 100µl 3X boiling mix. Samples were heated at 95°C for 15min, with occasional mixing, to shear the DNA and hence reduce the viscosity. 5µl samples were spotted on a Whatman No.1 disc for a trichloroacetic acid (TCA) wash in order to determine the percentage incorporation of [^{35}S]-methionine (See 3.11.3). Radiolabelled proteins were analysed by SDS-PAGE.

3.8.2 Late viral protein synthesis

HFL cells were infected with 20 p.f.u. virus at 37°C, in the absence of cycloheximide. Polypeptides were radiolabelled at 8h post-adsorption and harvested as previously described (See 3.8.1).

3.9 Preparation of cell extracts

3.9.1 Preparation of nuclear extracts

HeLa cell nuclear extracts were prepared using a modification of the procedure described by Dignam *et al.* (1983). Cells were washed with 10ml ice cold PBS and scraped into 5ml PBS. Cells were pelleted by centrifugation at 2,000 rpm for 5min in a Beckman GPR centrifuge and resuspended in 0.5ml PBS, before transfer to a 1.5 ml vial. Samples were centrifuged at 5,000 rpm in a benchtop microfuge for 2min. The supernatant was discarded and the cell pellet resuspended in 50µl buffer A, placed on ice for 5min, vortexed, returned to the ice for a further 5min, and vortexed again. The nuclei were pelleted by centrifugation at 4,000 rpm in a Sorvall SS34 rotor for 5min, the cytoplasmic fraction was removed and the nuclei packed down again by centrifugation at 15,000 rpm in a Sorvall SS34 rotor for 15min. Any remaining supernatant was removed and the nuclei were resuspended in 20µl buffer C. Samples were incubated on ice for 20min with frequent mixing and centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 15min to pellet the nuclear debris. The resulting supernatant was retained to be used as the nuclear extract, and the protein concentration determined (See 3.9.5).

3.9.1.1 Gel retardation assays

The reaction mixes used to analyse protein-DNA complex formation contained 10mM HEPES (pH 7.9); 0.6mM DTT; 2.3mM $MgCl_2$; 94mM NaCl; 0.1mM PMSF; 0.1mg/ml BSA; 4% v/v glycerol; 2µg poly(dI)-poly(dC); 0.2-1.0ng [^{32}P]-3'-end labelled DNA fragment and 5.0µg infected or mock infected HeLa cell extract. 0.3-1.0µg virion extract was added where appropriate. Samples were incubated at 25°C for 30min then loaded directly onto a 3.5% polyacrylamide gel. Electrophoresis was carried out in 0.5X TBE at 20mA for 3-4h. Gels were dried under vacuum, then exposed for autoradiography (Preston *et al.*, 1988).

3.9.2 Preparation of transcriptionally active whole cell extract

Whole cell HeLa extracts were prepared using the procedure described by Manley *et al.* (1980). All stages were carried out at 4°C. Cells were washed with 10ml ice cold PBS, before being scraped into 5ml PBS. The cells were pelleted at 2,000 rpm in a Beckman GPR centrifuge for 5min, and resuspended in four packed cell volumes (PCV) of 10mM Tris-HCl (pH7.9); 1mM EDTA, and 5mM DTT. After 20min the cells were lysed by homogenization in a Dounce homogeniser with eight strokes using a "B" type pestle. Four PCV of 50mM Tris-HCl (pH7.9); 10mM $MgCl_2$; 2mM DTT; 25% sucrose and 50% glycerol were added, and the suspension gently mixed. With continued gentle stirring, one PCV of saturated $(NH_4)_2SO_4$ was added dropwise to produce a highly viscous lysate. Stirring was continued for a further 30min and had to be gentle to prevent shearing of DNA, which would interfere with its removal at the next stage. This extremely viscous extract was carefully poured into polycarbonate tubes and centrifuged at 45,000rpm for 3h in a Beckman T_{100} rotor. The resulting supernatant was carefully decanted and the protein and nucleic acids were precipitated by the addition of solid $(NH_4)_2SO_4$ (0.33mg/ml solution). 1M NaOH (0.1ml/10mg solid $(NH_4)_2SO_4$) was added and the suspension stirred for another 30min. The precipitate was collected by centrifugation at 15,000 rpm in a Sorvall SS34 rotor for 20min, and resuspended in 5% of the volume of the high speed supernatant with 25mM HEPES (pH7.9); 100mM KCl; 12mM $MgCl_2$; 500mM EDTA; 2mM DTT and 17% glycerol. The suspension was dialysed against two changes of 50-100 volumes each of the resuspension buffer for a total of 8-12h. The dialysate was then centrifuged at 10,000 rpm for 10min in a Sorvall SS34 rotor to remove insoluble material. The supernatant was dispensed into 0.2-0.5ml portions, frozen on dry ice and stored at -70°C.

3.9.2.1 *In vitro* transcription assays (whole cell extract)

A typical 20µl reaction contained 60% (v/v) whole cell extract; 0.2-1.5µg template DNA; 50µM ATP; 50µM GTP; 50µM CTP; 5µM UTP; 5mM creatine phosphate; and 10µCi [α-^{32}P]UTP. The samples were incubated at 30°C for 2h and the reaction was terminated with the addition of 400µl of 0.3M sodium acetate; 10mM EDTA; 10mM Tris-HCl pH7.9; 0.5% SDS. 10µg yeast RNA was added then two phenol/chloroform and one chloroform extractions were carried out. Radiolabelled RNA was precipitated by the addition of 2.5vols ethanol and

storage at -20°C for at least 2h. Following a 70% ethanol wash, the RNA pellets were resuspended in 30µl 95% formamide; 0.5% 0.5X TBE. 1µl of each reaction was spotted onto a Whatman No.1 to determine the percentage incorporation of the radiolabel (See 3.11.3). The samples were heated for 2-3min on a heating block at 100°C, then loaded onto a 3.5% acrylamide; 7M urea gel. The gel was run at 150V for 3-4h, dried under vacuum, and exposed to autoradiographic film.

3.9.2.2 *In vitro* transcription assay-Promega nuclear extract

Mixtures containing (11-x)µl transcription buffer (where x=volume nuclear extract); 1.5µl 50mM $MgCl_2$; 0.4mM of each ATP, GTP and TTP; 4µl linear DNA template; 1µl [α-^{32}P]CTP (10mCi/ml) were assembled in a 1.5ml reaction vial. Nuclease free DW and 8units (xµl) of nuclear extract were added to bring the volume to 25µl. Reactions were briefly vortexed and incubated at 30°C for 60min. 175µl of stop solution was added to terminate the reaction. RNA was extracted with 200µl phenol:chloroform:isoamylalcohol (25:24:1), vortexed for 10sec, then the phases separated by centrifugation at 13,000 rpm for 5 min in a benchtop microfuge. The upper aqueous phase was transferred to a fresh tube and 100µl 100% ethanol was added. The samples were mixed by vortexing then placed on ice for 15min. RNA was pelleted by centrifugation at 13,000 rpm for 10min in a benchtop microfuge, the supernatant was decanted and the pellet dried in a vacuum desiccator. RNA was resuspended in 20µl nuclease free DW and 20µl Promega loading dye. Samples were heated to 90°C for 10min just prior to loading onto a gel.

3.9.3 Harvest and preparation of cell extracts for chloramphenicol acetytransferase (CAT) assays

The overlying medium was aspirated off using a Pasteur pipette and the cell monolayer washed with 2ml cold PBS. Cells were then scraped into 2ml TNE (10mM Tris-HCl; 100mM NaCl; 1mM EDTA (pH7.5)) and transferred to a sterile 15ml Falcon tube. A further 1ml TNE was added to the plate in order to remove any remaining cells. The cells were then pelleted by centrifugation at 2,000 rpm for 1min in a Beckman GRP centrifuge, and left in an inverted position to dry. 75µl of 250mM Tris-HCl pH7.8 was added and the tubes vortexed to resuspend the cells. Samples were sonicated for a series of 15sec bursts, until the suspension appeared slightly opaque. Samples were

transferred to a 1.5ml reaction vial and the cell debris pelleted by centrifugation at 13,000 rpm in a benchtop microfuge for 2min. The resulting supernatant contained CAT activity and was stored at -70°C.

3.9.3.1 CAT assays

Each reaction contained 1µl 50mM acetyl coenzyme A; 0.5µl stock ^{14}C-chloramphenicol; 14µl DW and 25µl of cell extract. The reactions were initiated by vortexing and incubated for 1h at 37°C. Reactions were stopped by the addition of 200µl ethyl acetate. After vortexing briefly the tubes were centrifuged at 13,000 rpm for 1min in a benchtop microfuge. The upper layer was transferred to a fresh tube, and vacuum dried. The pellet was dissolved in 20µl ethyl acetate by vortexing. Samples were spotted on a thin layer chromatography (TLC) plate, no closer than 1cm apart and 2cm from the base. The TLC plate was placed in a chromatography tank containing 5% methanol; 95% chloroform. Once the elutent reached 1cm from the top, the plate was removed and left to air dry. When completely dry it was exposed directly to photographic film at RT.

3.9.4 Preparation of cell extracts for thymidine kinase (TK) assays

Cells were scraped into the medium, then pelleted by centrifugation at 5,000 rpm for 15min in a benchtop microfuge. The pellet was vortexed and 0.1ml TK lysis buffer was added. The samples were vortexed, then left on ice for 5-10min. After a second vortex, they were centrifuged for 2min at 13,000 rpm in a benchtop microfuge. The resulting supernatant contained the extract to be assayed for TK activity.

3.9.4.1 Determination of TK activity

Each TK reaction mix contained 50mM sodium phosphate buffer (pH6.0); 10mM $MgCl_2$; 5mM ATP; 0.1mM dTTP; 100µCi/ml [^3H]-thymidine. 5µl of cell extract was added to 45µl of the above reaction mix, and the samples vortexed. Incubation was carried out at 30°C for 1h. 10µl 100mM EDTA; 1mM thymidine was added and the samples heated for 5min at 100°C, and cooled on ice for 5min. They were then centrifuged for 5 min at 13,000 rpm in a benchtop microfuge and 50µl of the supernatant was spotted onto a DE8I disc. The discs were immersed in 500ml 4mM ammonium formate; 10µM thymidine

at 37°C for 10min. The first wash was poured off and another 500ml added. This procedure was repeated a third time, followed by two 5min ethanol washes. The discs were placed out to air dry, and the amount of ^{3}H-thymidine incorporated was determined in a scintillation counter (See 3.11.3).

3.9.5 Protein concentration estimations

The protein concentrations of sample was determined using a Bradford assay (Bradford, 1976). 1ml of Bradford reagent was added to a sample of the cell extract and left at RT for 15min. The absorbance was determined at 595nm and the protein concentration estimated from a calibration curve derived from known concentrations of BSA.

3.10 Manipulation of oligonucleotides

3.10.1 Purification of oligonucleotides

Deprotection of oligonucleotides was achieved by incubation at 55°C for 5h. After dispensing into four aliquots, they were frozen on dry ice then dried under vacuum for 1.5h. Each pellet was resuspended in 50µl of 90% formamide (deionised); 1X TBE, heated at 95°C for 2min before being applied to a 15% acrylamide gel. The desired band was detected by placing a TLC plate underneath the gel and illuminating it with short wave UV light. The oligonucleotide appeared as a distinct grey band on a green background. The uppermost band contained the full length oligonucleotide, and was excised from the gel and incubated at 45°C overnight in 2ml gel elution buffer (GEB). The content of each tube was filtered through glass fibre into a fresh vial and ethanol precipitated as previously described. Oligonucleotide pellets were stored dry at -70°C and resuspended in DW when required.

3.10.2 Annealing complementary oligonucleotides

5µg of each oligonucleotide was brought up to 50µl in a solution containing 0.3M sodium acetate; 2mM EDTA; 40mM Tris-HCl pH7.5. Samples were incubated at 50°C for 1h, 37°C for 1h and RT for 1h. Annealed oligonucleotides were precipitated in 2.5 volumes of ethanol at -20°C for 2h and pelleted by centrifugation at 13,000 rpm in a benchtop microfuge for 20min. After a 70% ethanol wash, the pellet was dried under vacuum and resuspended in the required volume of DW.

3.11 ^{32}P-labelling of DNA probes *in vitro*

3.11.1 Hexanucleotide primer extension

1µg of DNA was dissolved in a total volume of 23.5µl of sterile DW and denatured by heating at 100°C for 10min. The sample was cooled on ice for 10min, before a brief centrifugation to recover water lost by condensation. To this, 10µl oligo reaction mix; 20µM each non-radioactive dATP, dGTP, dTTP; 2.0µl 0.1% BSA; 5.0µl (50µCi) α-[^{32}P]-dCTP and 5 units Klenow enzyme were added. Reactions were incubated at RT overnight (Feinberg & Vogelstein, 1983).

3.11.2 End-filling labelling of oligonucleotide probes

A typical 50µl reaction contained 15-20mM oligonucleotide; 1X T4 pol buffer; 1mM DTT; 0.1mM each non-radioactive dATP, dGTP; 2.5µl (25µCi) each of α-[^{32}P]-dCTP, dTTP; 1/10 volume 0.1% BSA and 5-10 units T4 DNA polymerase. The samples were incubated at 37°C for 1h. Upon completion of the reaction, 15µl of TBE loading buffer was added and the entire sample loaded directly onto a 8% native polyacrylamide gel. Electrophoresis was carried out at 150V until the BPB band reached approximately 2 inches from the bottom. The highly radioactive bands were identified by autoradiography of the wet gel, excised and eluted at 45°C overnight in 0.5ml GEB. The supernatant was purified by passing it through a 10ml Sephadex G50 column. The peak, usually four four drop fractions, was collected and 1µg poly(dI).poly(dC) was added to each, along with sodium acetate to 0.3M and 2.5 vols ethanol. After at least 2h at -20°C, the oligonucleotides were pelleted by centrifugation, washed in ethanol, then dissolved in 50µl DW. 1µl of this was removed and its Cerenkov c.p.m. was determined.

3.11.3 Determination of percentage incorporation of radiolabel into probe

0.5µl of the above labelling mix was diluted into 10µl DW. 3µl duplicate samples were spotted onto Whatman No.1 filter paper discs. The filters were dried briefly under a heat lamp, and one kept aside to determine the total c.p.m. in the sample. In order to remove the unincorporated radiolabelled nucleotides, the other filter was placed in 10% (w/v) TCA for 5min, then transferred to a fresh 10% (w/v) TCA wash for a further 5min. Two 5min 5% (w/v) TCA washes were then carried out followed by two 5min ethanol

washes. The discs were dried then placed in a scintillation vial containing 5ml Ecoscint A. The level of radioactivity was measured in a scintillation counter and compared to that of the unwashed sample.

3.12 Southern transfer and hybridisation

3.12.1 Southern blot-alkaline transfer method

The trimmed agarose gel was soaked in 50ml of alkaline transfer solution for 30min with occasional agitation. Two pieces of Whatman No.1 filter paper and one piece of Genescreen Plus membrane were cut to the same size as the gel. Two filter paper wicks were soaked in alkaline transfer solution, and placed over a glass plate on a rack with the ends touching the bottom of the dish. It was important to ensure that there were no air bubbles as this would interfere with the DNA transfer. The gel containing denatured DNA was placed on top of the wicks, the Genescreen Plus membrane and two pieces of filter paper were placed on top of the gel. A 2 inch thick pile of paper towels were added and weighted down. The remaining alkaline transfer solution was poured into the dish and left for 16-18h to allow capillary transfer to occur. The membrane was immersed in 200ml of neutralising solution for 15min and air dried. The DNA was irreversibly bound to the membrane by exposing it to short wave UV light for 6min (Chomczynski & Qasba, 1984).

3.12.2 DNA hybridisation with DNA probes

The Genescreen Plus membrane was prehybridised in 100ml of prehybridisation mix for 3h at 72°C in a shaking water bath. The prehybridisation mix was poured off and 20ml hybridisation mix containing the radiolabelled probe was added using a syringe and needle. The bag was resealed and the blot returned to the shaking 72°C water bath overnight. The next morning two 45min washes in prehybridisation mix were carried out at 72°C. The blot was finally rinsed quickly in DW at RT then left to air dry, before autoradiography.

3.13 RNA blot transfer and hybridisation

3.13.1 RNA dot blots

RNA samples were diluted to 3, 1 and 0.3µg in a total volume of 50µl. 9 X 12cm pieces of nitrocellulose and Whatman No.1 filter paper were cut and soaked in DW and 20 X SSC, before being placed on a hybrid manifold with the filter paper beneath the nitrocellulose. The apparatus was then reassembled and the plates screwed in position. 50µl dilutions of each sample was loaded into the appropriate well under suction. The water pressure was increased to full pressure for 5min. Once the apparatus was dismantled, the nitrocellulose filter was placed on a tissue and left to air dry overnight at RT. Filters were baked in a vacuum oven for 2h at 65°C prior to hybridization (White & Bancroft, 1982).

3.13.2 Northern transfer from formaldehyde/agarose gels

The formaldehyde/agarose gel was prepared for northern transfer by washing twice in DW for 5min then equilibrating in 10X SSC for 45min. The blot was set up as described for the Southern blot, but using a nitrocellulose filter and transferring in 20X SSC. The filter was prehybridised at 42°C for 2-4h in 50% formaldehyde (deionised); 5X SSPE; 5X Denhardt's solution; 0.5% SDS and 100µg/ml denatured and sonicated calf thymus (CT) DNA. Hybridisation was carried out using fresh prehybridisation buffer containing denatured and neutralised radiolabelled probe, overnight at 42°C. The filter was washed for 20min in 2X SSC; 0.1% SDS at 65°C, for 10min in 0.2X SSC; 0.1% SDS and for 10min in 0.2X SSC.

3.13.3 RNA hybridisation with DNA probes

Nitrocellulose filters were prehybridised for 3h at 42°C in 15ml of 50% formamide (deionised); 5X SSPE; 5X Denhardt's solution and 100µg/ml sonicated denatured calf thymus DNA. Hybridisation was carried out at 42°C in 8.8ml 50% formamide (deionised); 5X SSPE; 1X Denhardt's solution; 100µg/ml CT DNA and a radiolabelled DNA probe. After incubation for 16h the hybridisation solution was removed and two 30min washes in 2X SSC, 0.1% SDS at RT; two 30min washes in 0.1X SSC, 0.1% SDS at RT and two 15min washes in 0.1X SSC, 0.1% SDS at 60°C were carried out.

3.14 Preparation of HMBA stock solution

A stock solution of 100mM HMBA was prepared in PBS by incubation at 37°C for 1h, and filter sterilised before use.

*in1814 particle counts were obtained by electron microscope analysis and the corresponding particle to pfu ratio was determined. The isolates of in1814 used in this thesis all had the same particle to pfu ratio.

4. RESULTS

4.1 Objective

A number of structurally and functionally related chemicals have been identified which reverse the phenotype of the Vmw65 insertion mutant, *in*1814 (Ace *et al.*, 1989) in tissue culture cells, such that replication rather than latency is the normal outcome of infection (McFarlane *et al.*, 1992). The work presented in this thesis documents the characterization of the effect of HMBA on *in*1814 replication, the investigation into how HMBA functions at the molecular level and the elucidation of the signals involved in this process. HMBA and DMSO are both potent inducers of terminal differentiation of MELCs (Friend *et al.*, 1971; Reuben *et al.*, 1976) and have been shown to increase the speed and efficiency of reactivation of both HSV-1 and HSV-2 from the latent state in a number of animal models (Whitby *et al.*, 1987; Bernstein & Kappes, 1988; Leib *et al.*, 1989). Determination of the molecular basis for the activity of HMBA on *in*1814 infection may reveal the primary events by which the compound affects the poorly understood process of reactivation. In addition, stimulation of HSV-1 IE RNA accumulation is, to our knowledge, the first example in which genes in their normal genomic environment are activated by HMBA, and therefore studies on the mechanism of the effect on IE gene expression should be relevant to understanding the primary changes in gene expression that trigger erythroleukaemic cell differentiation.

4.2 The effects of HMBA on *in*1814 replication

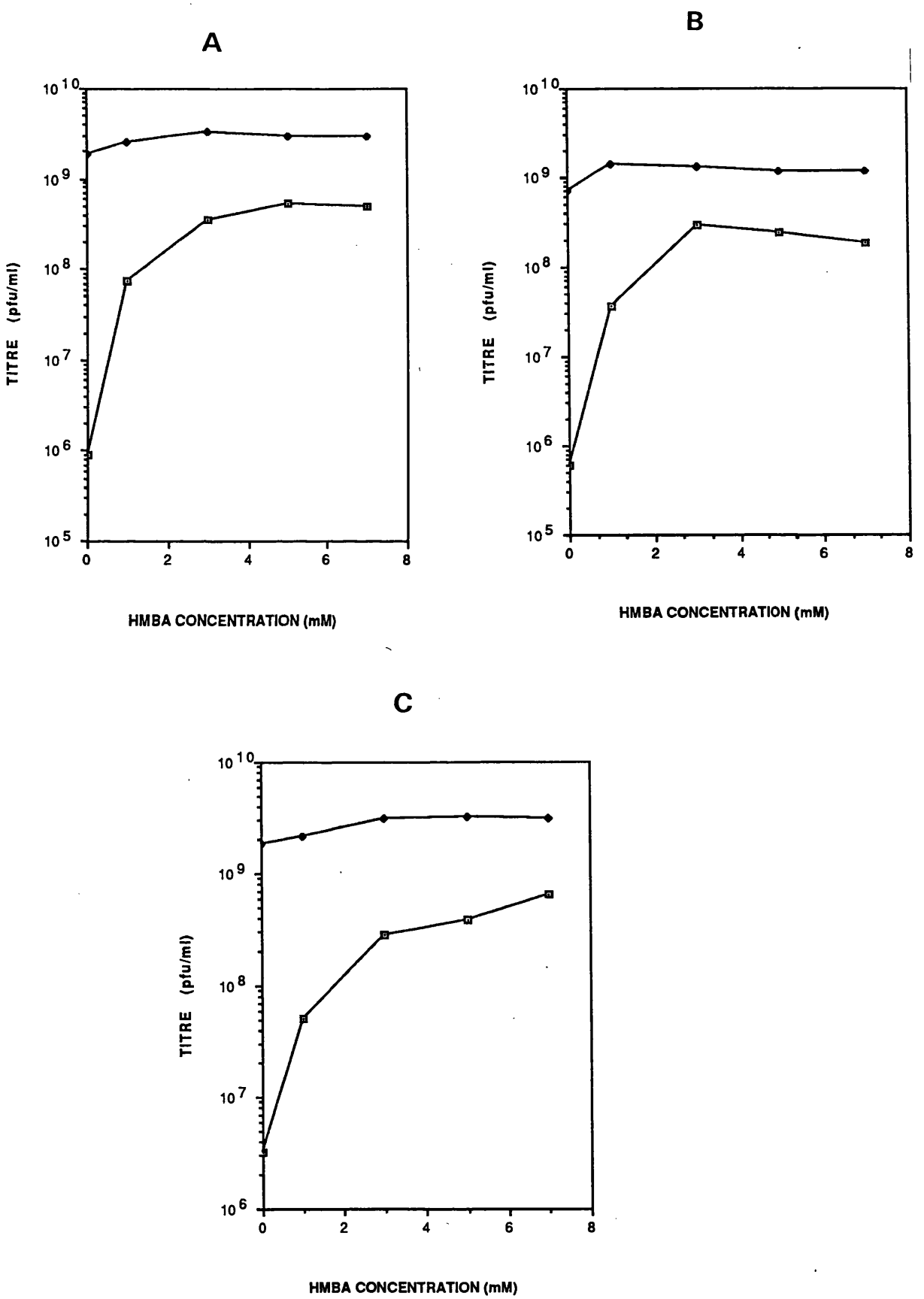
4.2.1 Effect of HMBA on *in*1814 titre

*Titration of *in*1814, and the rescued 'revertant' 1814R, was carried out on HFL cells, with various concentrations of HMBA included in the culture medium after a 1h adsorption and penetration period. Tenfold dilutions of virus suspension (200µl) were added to cell monolayers on 30mm diameter dishes. After incubation for 1h, to allow adsorption and penetration of virus, 2ml of EHu5 was added. HMBA was routinely added after the 1h period. A large increase in *in*1814 titre was observed with increasing concentrations of HMBA, reaching a value of 500-fold at the optimal concentration, 5mM HMBA (Figure 4.1a). Titration of *in*1814 in the presence of 5mM HMBA reduced the

Figure 4.1 Effect of HMBA on the titre of *in*1814 and 1814R

Mutant *in*1814 (■) and wt revertant 1814R (◆) were titrated on HFL (a), BHK (b) or HeLa (c) cells. Monolayers were fixed and stained 2 days post-infection.

Figure 4.1


A
B
C
TITRE (pfu/ml)
HMBA CONCENTRATION (mM)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
2
4
6
8

particle to pfu ratio from 30,000 to 60, a value close to that routinely observed for wild type HSV-1 preparations. In contrast, the titre of 1814R was virtually unaffected by the presence of HMBA. At 5mM HMBA, HFL cells were slightly elongated but otherwise morphologically normal, whereas evidence of cell degeneration was observed at 7mM HMBA.

To determine whether the effect of HMBA was specific to HFL cells, *in*1814 and 1814R were titrated on BHK and HeLa cells in the presence and absence of HMBA (Figure 4.1b,c). Again *in*1814 reached a titre of 5 x 10^8 pfu/ml or greater, raising the possibility that HMBA is either affecting or working via a ubiquitous phenomenon or protein.

The effect of HMBA on overall protein synthesis after infection of HFL cells was investigated at a relatively low m.o.i. as the detrimental effect of the Vmw65 mutation on viral replication is not observed at high m.o.i. (Ace *et al.*, 1989). HFL monolayers were infected, in the absence of cycloheximide, with 10 particles of *in*1814 or 1814R per cell, in the presence or absence of 5mM HMBA, and radiolabelled after 8h with [^{35}S]-methionine (Figure 4.2). The presence of HMBA did not significantly alter the polypeptide profile of mock- (lanes 1 and 2), or 1814R-infected (lanes 3 and 4) cells, but changed the pattern of *in*1814-infected cells (lanes 5 and 6) to resemble that of 1814R-infected cells rather than mock-infected cells.

The presence of 3-5mM HMBA, the optimum concentration for induction of MELC differentiation (Reuben *et al.*, 1980), therefore increases dramatically the titre of *in*1814, enabling the mutant to initiate infection almost as efficiently as wild type HSV-1.

4.2.2 Effects of DMSO, hypoxanthine and 5-azacytidine on *in*1814 titre

The effects on the titre of *in*1814 of two other agents known to induce erythroid differentiation was investigated (Table 4.1). DMSO, like HMBA, is a polar-planar compound whereas hypoxanthine is not strongly charged. Both compounds significantly increased *in*1814 titre at concentrations previously shown to be optimal for differentiation (Gusella *et al.*, 1976; Reuben *et al.*, 1980), but they were not as effective as HMBA. This result is consistent with the observation by Palfrey *et al.* (1977) that HMBA is a more potent inducer of MELC differentiation, acting in a concentration range 50 times lower than that of DMSO.

The increased efficiency and speed of reactivation of latent DNA in the presence of HMBA or DMSO in a number of different model systems

Figure 4.2 Effect of HMBA on HSV-1 protein synthesis

HFL monolayers were mock-infected (lanes 1 and 2), infected with 10 particles of 1814R (lanes 3 and 4) or *in*1814 (lanes 5 and 6) per cell, in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 5mM HMBA. At 8h post-infection, monolayers were radiolabelled with [^{35}S]methionine, in the absence of HMBA. The positions of virus-specified proteins Vmw155, glycoprotein B and Vmw65 are shown.

Cells were labelled for 90 min in the presence of 1mg/ml actinomycin D.

The apparent increased molecular weight of Vmw65 specified by *in*1814, due to the insertion of 4aa, has been previously documented by Ace *et al.*, 1989.

Figure 4.2

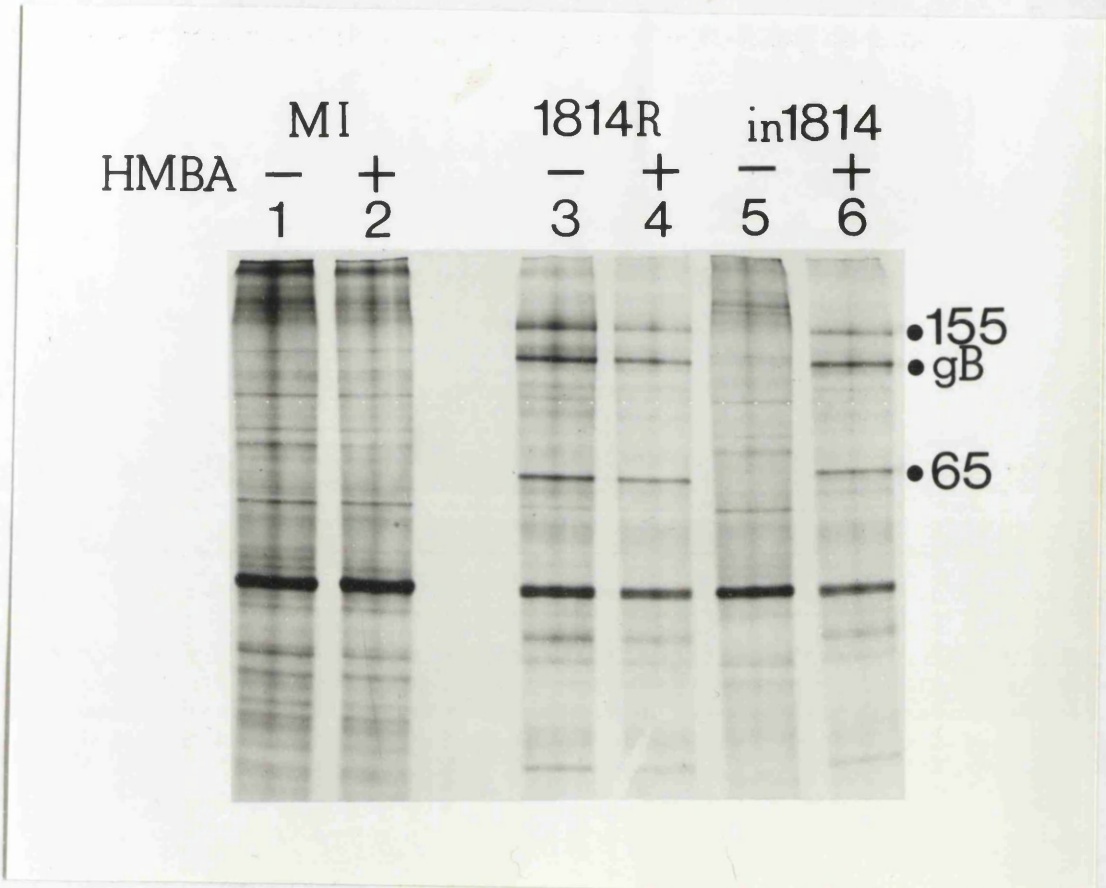


Table 4.1 Effect of DMSO and Hypoxanthine on the titre of *in*1814

Virus	Treatment	Titre (pfu/ml)
*in*1814	NONE	$<5 \times 10^5$
*in*1814	5.0mM HMBA	1.2×10^9
*in*1814	100mM DMSO	1.4×10^8
*in*1814	150mM DMSO	2.7×10^8
*in*1814	200mM DMSO	4.4×10^8
*in*1814	2.5mM Hypoxanthine	3.5×10^8
*in*1814	3.75mM Hypoxanthine	3.9×10^8
*in*1814	5.0mM Hypoxanthine	5.3×10^8
1814R	NONE	1.3×10^9
1814R	5.0mM HMBA	1.2×10^9
1814R	200mM DMSO	1.2×10^9
1814R	5.0mM Hypoxanthine	1.2×10^9

HFL cells were infected with either *in*1814 or 1814R and the agents added to the culture medium after the 1h adsorption and penetration period. Monolayers were fixed and stained two days post-infection and the resulting titres determined.

(Whitby *et al.*, 1987; Bernstein & Kappes, 1988; Leib *et al.*, 1989) has been attributed to demethylation, for two reasons. Firstly, addition of 5-azacytidine or L-ethionine also improved reactivation of latent HSV (Whitby *et al.*, 1987; Stephanopoulos *et al.*, 1988), and secondly, during induction of MELCs, a transient decrease in 5-methycytosine levels occurs (Razin *et al.*, 1988). Therefore to determine whether demethylation is the underlying mechanism whereby HMBA exerts its effect on *in*1814, titrations of *in*1814 and 1814R were carried out in the presence of the hypomethylating agent 5-azacytidine (Table 4.2). To determine whether 5-azacytidine could directly antagonize the HMBA effect on *in*1814 titre, cells were exposed to both 5mM HMBA and 8μM 5-azacytidine after the 1h adsorption and penetration period until 48h, when the cells were fixed and stained and the resulting titres determined. In addition, HFL cells were pretreated with 8μM 5-azacytidine for 24h and the agent was either retained after adsorption or withdrawn immediately prior to infection. Addition of 8μM 5-azacytidine decreased the titre of *in*1814, especially when present prior to and after the addition of virus, whereas little effect was noted on the titre of 1814R (Table 4.2). Therefore, hypomethylation of DNA by exposure of cells to HMBA is not responsible for the effect on the replication of the HSV-1 Vmw65 insertion mutant *in*1814.

4.2.3 HMBA exerts its effect transiently, early during infection

For the experiments shown in Figure 4.1, HMBA was present from 1h after the addition of virus until monolayers were stained, usually two days later. The time of exposure required for maximum effect was subsequently investigated. It was first ascertained that pre-exposure of cells to 5mM HMBA for 1h to 5h immediately prior to infection gave no stimulation of plaque numbers, provided the monolayers were washed thoroughly before the addition of virus (results not shown), demonstrating that HMBA does not act by inducing stable cellular alterations.

The time of exposure after infection was therefore varied (Figure 4.3). Mutant *in*1814 was titrated on HFL cells, and EHu5 containing 5mM HMBA was added after adsorption and penetration. At various times, the medium was removed, monolayers were washed twice with EHu5 and overlaid with EHu5. Cells were fixed and stained 2 days later and the resulting titres determined. It was found that only 1h of treatment with HMBA gave a large increase in titre, and that retaining the agent until 5h post-infection was as effective as

Table 4.2 Efffect of 5-azacytidine on the titre of *in*1814 and 1814R

5-azacytidine (μM)		Titre (pfu/ml)	
Pre-infection[a]	**Post-infection[b]**	***in*1814**	**1814R**
0	0	3.5×10^6	2.8×10^8
8	0	2.2×10^6	2.8×10^8
0	8	7.5×10^5	3.0×10^8
8	8	$<5 \times 10^4$	1.7×10^8

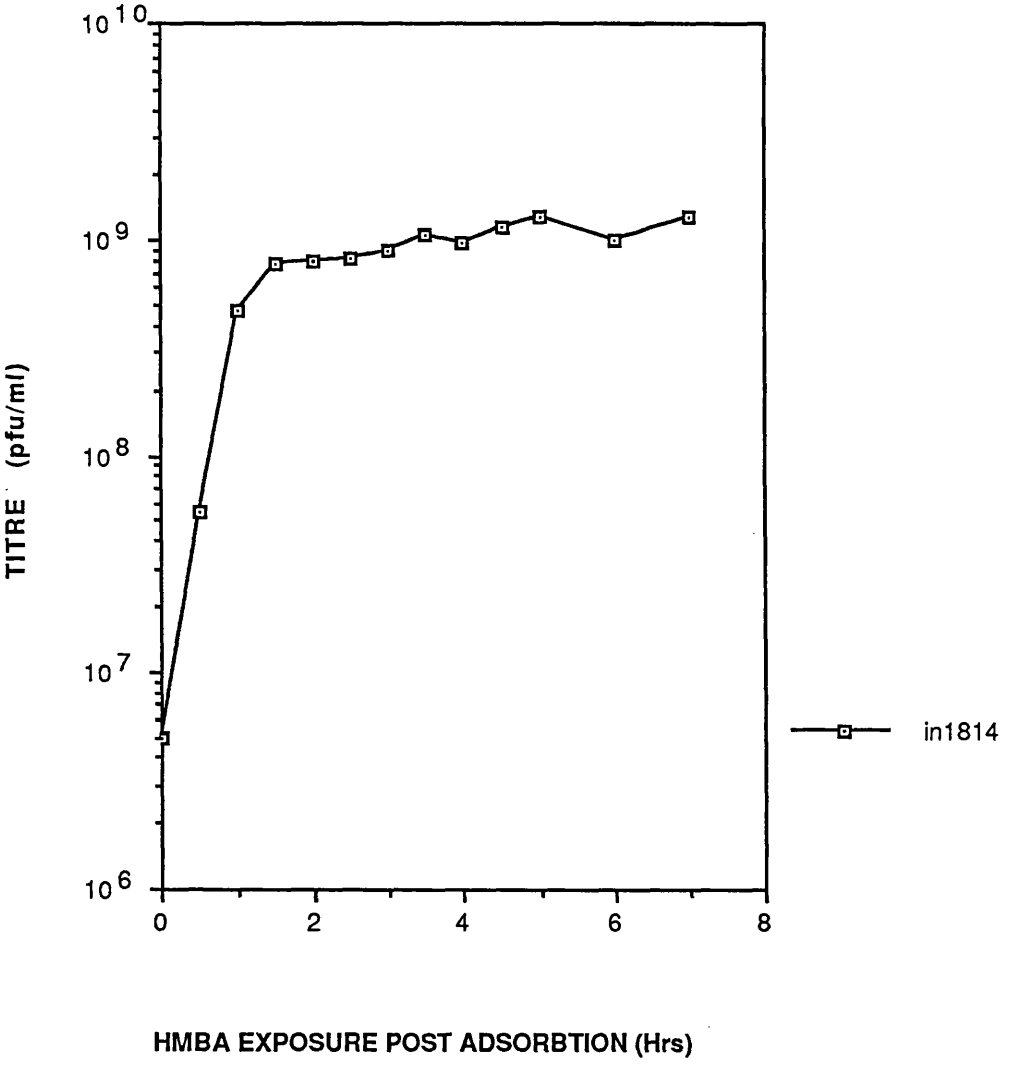
a 5-azacytidine present for 24h immediately prior to infection.

b 5-azacytidine present after adsorption and penetration.

Figure 4.3 Time of exposure to HMBA required for maximum increase in titre

Mutant *in*1814 was titrated on HFL cells, and EHu5 containing 5mM HMBA was added after adsorption and penetration. At various times, the medium was removed, monolayers were washed twice with EHu5 and overlaid with EHu5. Monolayers were fixed and stained 2 days post-infection.

Figure 4.3


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
TITRE (pfu/ml)
0
2
4
6
8
HMBA EXPOSURE POST ADSORBTION (Hrs)
in1814

maintaining it throughout the 48h titration period. HMBA therefore exerts its effect early during infection, and is only required transiently.

The time of addition of HMBA after infection was the next variable investigated (Figure 4.4). HMBA was added to the culture medium at various times after adsorption and penetration of *in*1814. A steady decline in the response was observed and by 6h after infection the addition of HMBA was without effect. Cells infected with *in*1814 thus rapidly became refractory to activation of the viral genome by HMBA. The loss of responsiveness of *in*1814 to HMBA is reminiscent of the progressive insensitivity to complementation by Vmw65 observed previously by Harris and Preston (1991). In the absence of sufficient IE protein production the *in*1814 genome is converted to a latent state in which it is refractory to the action of all trans-activator proteins except Vmw110 (Harris & Preston, 1991).

4.2.4 Addition of HMBA stimulates IE RNA production

The finding that HMBA raises the titre of *in*1814, which is known to synthesize reduced levels of IE RNAs (Ace *et al.*, 1989), and that it acts early in infection, suggests that the agent acts by increasing the amounts of IE RNAs, and hence IE proteins. HFL cells were infected with 100 particles of *in*1814 or 1814R per cell and incubated for 5h in the presence of 25μg/ml cycloheximide, with or without the addition of 5mM HMBA. Cytoplasmic RNA was isolated and analysed by dot blot hybridization, using radiolabelled IE gene-specific probes (Figure 4.5) representing IE-1, IE-2, IE-3 and IE-4. As found after infection of BHK cells (Ace *et al.*, 1989), *in*1814-infected HFL cells accumulated lower levels of IE RNAs than 1814R-infected cells (Figure 4.5). Incubation with 5mM HMBA resulted in an increase of approximately twofold in IE RNA accumulation, bringing the levels close to those found in untreated, 1814R-infected cells. The greatest increase was seen for Vmw110 and Vmw63 RNA levels, with a smaller increase in Vmw68 and Vmw175 RNAs. These increases are consistent with the observed phenotype of *in*1814, which shows a fourfold reduction in the levels of accumulation of Vmw110 and Vmw63, a twofold reduction in Vmw68 RNA but little effect on the level of Vmw175 (Ace *et al.*, 1989). The level of cellular actin RNA was not significantly affected by HMBA. The effect of HMBA on the titre of *in*1814 can thus be explained by an increase in IE RNA accumulation. HMBA can therefore exert its effect in the absence of protein synthesis, suggesting that its target may be a pre-existing cellular or virus structural component(s).

Figure 4.4 Time of effectiveness of HMBA

Mutant *in*1814 was titrated on HFL monolayers and HMBA was added to a final concentration of 5mM at the times after adsorption and penetration indicated. Monolayers were fixed and stained 2 days post-infection. In the absence of exposure to HMBA, the titre of *in*1814 on HFL cells is 5×10^6.

Figure 4.4

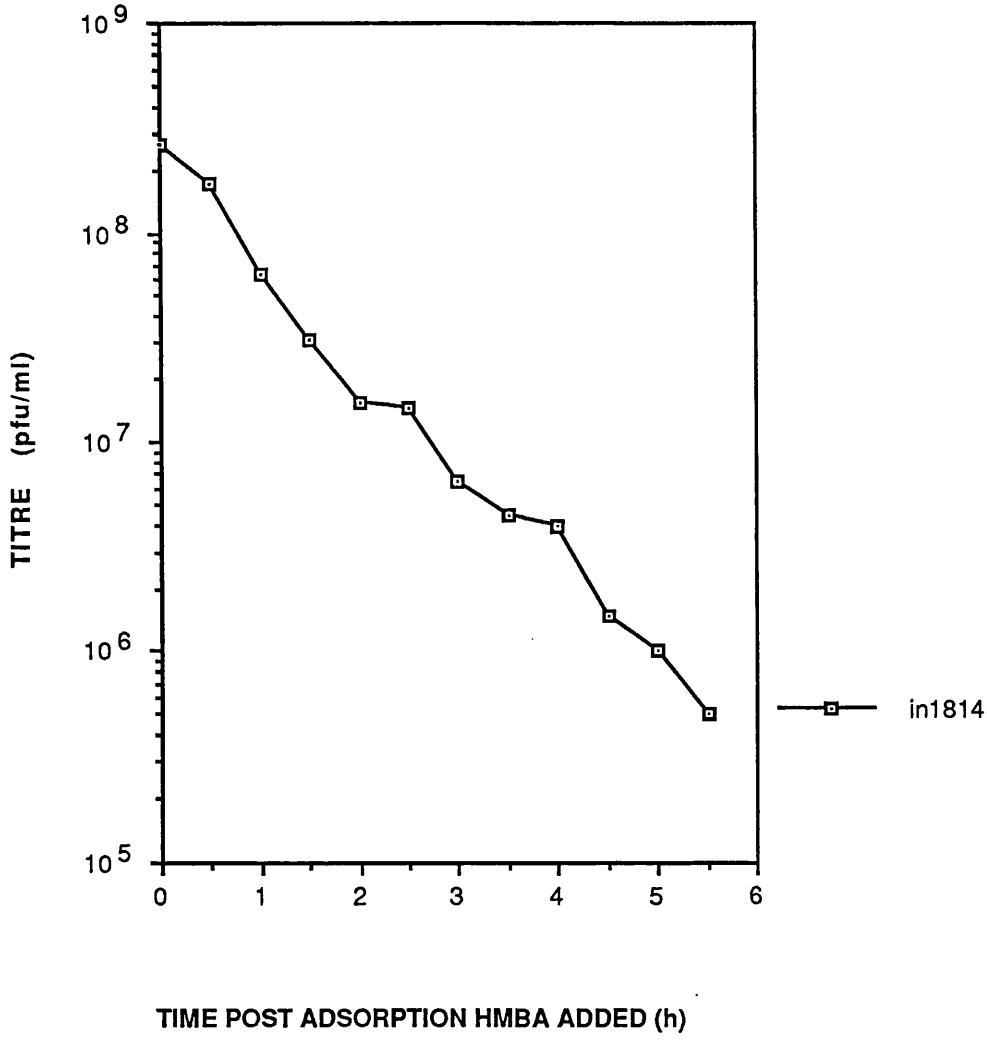

10^9
10^8
10^7
10^6
10^5
TITRE (pfu/ml)
0
1
2
3
4
5
6
TIME POST ADSORPTION HMBA ADDED (h)
in1814

Figure 4.5 Effect of HMBA on IE RNA accumulation

HFL cells were infected with 100 particles of *in*1814 (14) or 1814R (R) per cell, or mock-infected (M), in the presence of 25µg/ml cycloheximide. After adsorption and penetration, culture medium containing cycloheximide and, where appropriate, 5mM HMBA was added. After a further 4h, RNA was extracted and 3, 1 or 0.3µg was applied to nitrocellulose membranes. Membranes were probed with radiolabelled DNA fragments specific for IE genes 1 (encodes Vmw110), 2 (encodes Vmw63), 3 (encodes Vmw175) or 4 (encodes Vmw68), or mouse γ-actin (A).

The absorbance at 260nm was measured to determine the concentration of the RNA.

This experiment has been repeated, and the results obtained here confirmed. This effect has also been demonstrated in BHK cells and the levels of the IE RNAs, in the presence and absence of HMBA, has been determined by densitrometric analysis and normalized to actin (McFarlane *et al.*, 1992).

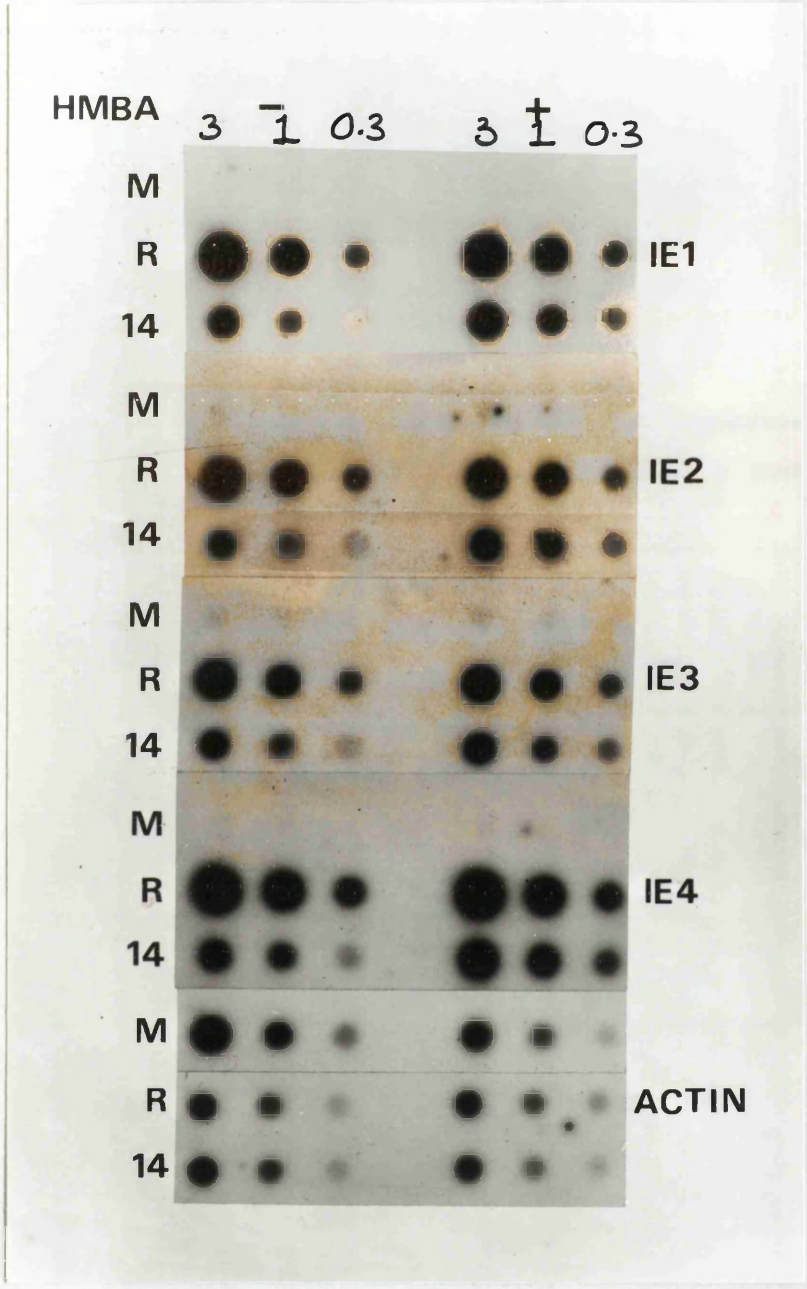
Hybridisation signals in figure 4.5 were quantified by densitometric measurement of spot intensities. The percent area under the curves after gaussian integration analysis are the values noted.

	-HMBA			+HMBA						
	3.0	1.0	0.3	3.0	1.0	0.3µg RNA	Mean Fold Increase			Mean Increase
1814R										
IE1	21.6	19.0	6.4	28.7	18.2	6.2	1.3	0.96	0.97	1.1
IE2	22.7	21.5	11.7	17.9	19.2	7.5	0.97	0.89	0.64	0.77
IE3	18.0	19.7	9.4	26.9	17.7	8.4	1.5	0.9	0.9	1.1
IE4	24.0	14.5	5.6	28.8	18.4	8.7	1.2	1.3	1.6	1.37
Actin	28.7	17.8	5.5	26.7	14.8	6.5	0.9	0.8	1.2	0.97

	-HMBA			+HMBA						
	3.0	1.0	0.3	3.0	1.0	0.3µg RNA	Mean Fold Increase			MeanIncrease
***in*1814**										
IE1	24.4	11.3	3.3	35.5	19.9	7.6	1.45	1.76	2.3	1.8
IE2	20.9	12.2	5.0	30.3	23.6	7.8	1.45	1.9	1.56	1.6
IE3	27.6	14.0	5.2	32.5	14.1	6.5	1.18	1.0	1.25	1.14
IE4	19.9	12.0	4.2	31.2	21.0	11.7	1.57	1.75	2.8	2.04
Actin	32.3	19.7	5.5	26.9	11.5	5.1	0.83	0.58	0.93	0.78

	-HMBA			+HMBA						
	3.0	1.0	0.3	3.0	1.0	0.3µg RNA	Mean Fold Increase			MeanIncrease
Mock										
Actin	24.2	22.4	9.2	27.1	13.2	3.9	1.12	0.59	0.42	0.71

Figure 4.5



4.2.5 HMBA does not affect transport of viral genomes to the nucleus

To investigate whether HMBA increased transport of *in*1814 DNA to the nucleus, HFL monolayers were infected with 100 particles of *in*1814 per cell in the presence of 25μg/ml cycloheximide, and cultured with or without 5mM HMBA. Nuclear DNA was extracted after 3h, cleaved with *Bam*HI, blotted and probed with a mixture of radiolabelled pTK1 and pMC17 (Figure 4.6). The intensity of hybridization to the three expected bands (5.0 and 3.0kbp from pMC17; 3.5kbp from pTK1) was unaffected by the presence of HMBA in the culture medium, demonstrating that HMBA does not increase the transport of *in*1814 DNA to the nucleus.

4.3 Specificity of action of HMBA

4.3.1 Effect of HMBA on promoters in plasmid based assays

To explore the responsiveness of a range of promoters and to potentially identify sequences that confer inducibility, CAT reporter gene constructs linked to various viral promoters were introduced into HFL cells by lipofection, in the presence and absence of HMBA. 1μg plasmid DNA was introduced into HFL cells, and the lipofection treatment carried out for 5h, at which time reactions were terminated with the addition of 2ml fresh EF_{10}. Cell extracts were prepared after 24h and CAT assays were carried out using equivalent amounts of protein (30μg) in each reaction for 1h at 30°C (Figure 4.7). All the plasmids tested showed increased CAT activity in the presence of 5mM HMBA. The results demonstrate an increase in CAT activity from IE, E and L HSV promoters, from the HCMV enhancer, and from the SV40 promoter and enhancer. The results reveal an apparent lack of promoter specificity in the mode of action of HMBA, as all the constructs tested in this transient system responded to HMBA treatment. Campbell *et al.* (1990) previously demonstrated that the HSV TK and SV40 early promoters were inducibile by HMBA in erythroleukaemic cells. However, constructs containing the mouse mammary tumour virus long terminal repeat (LTR), and the Moloney murine sarcoma virus LTR both failed to give significant increases in expression upon induction (Campbell *et al.*, 1990). Due to the inability to demonstrate any correlation between upstream promoter sequences and the activation of CAT activity by HMBA, Campbell *et al.* (1990) suggested that HMBA-mediated

Figure 4.6 HMBA does not affect transport of *in*1814 DNA to the cell nucleus

HFL cells were infected with 100 particles of *in*1814 per cell in the presence of 25μg/ml cycloheximide. After adsorption and penetration, medium containing cycloheximide and, where appropriate, 5mM HMBA was added. After a further 2h, nuclear DNA was extracted, cleaved with *Bam*HI and analysed by Southern hybridization. DNA was extracted from infected cells after incubation without (lane 1) or with (lane 3) HMBA or from mock-infected cells without HMBA (lane 2). (a) Ethidium bromide-stained gel; (b) autoradiograph.

This experiment has been repeated, and the results obtained here confirmed.

Hybridisation signals representing the 3.5 and 3.0kb bands in figure 4.6b were quantitated by densitometric measurement of spot intensities. The areas under the curves after gaussian integration analysis are 6298 in the presence of HMBA and 6262 in the absence of HMBA.

Figure 4.6a

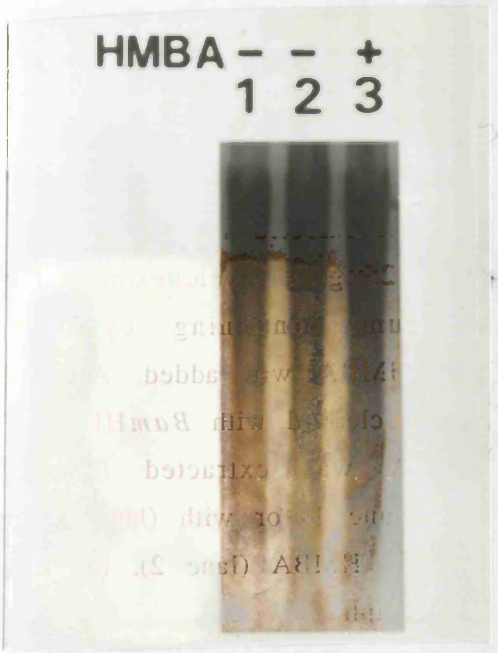


Figure 4.6b

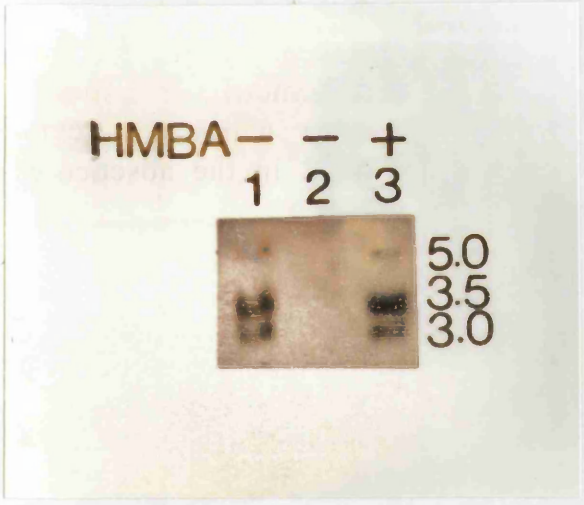


Figure 4.7 Effect of HMBA on different promoters in transfection assays

0.1µg of pLWP15 containing the IE-4 promoter minus the TAATGARAT motif (IE-4-), pLWUS containing the IE-4 promoter including the TAATGARAT motif (IE-4+), pIE1CAT containing the Vmw110 promoter (IE-1), pIE3CAT containing the IE-3 promoter (IE-3), pTKCAT containing the TK promoter (TK), pgDCAT containing the gD promoter (gD), pRR55 containing the HCMV IE promoter (HCMVIE) and pSVECAT containing the SV40 promoter (SV40) were introduced into HFL cells by a 5h lipofection treatment, in the presence (+) or absence (-) of 5mM HMBA in the culture medium. Cell extracts were prepared at 24h and CAT assays carried out at 30°C for 1h, then subjected to TLC. Plasmid constructs are described in detail in section 2.4.

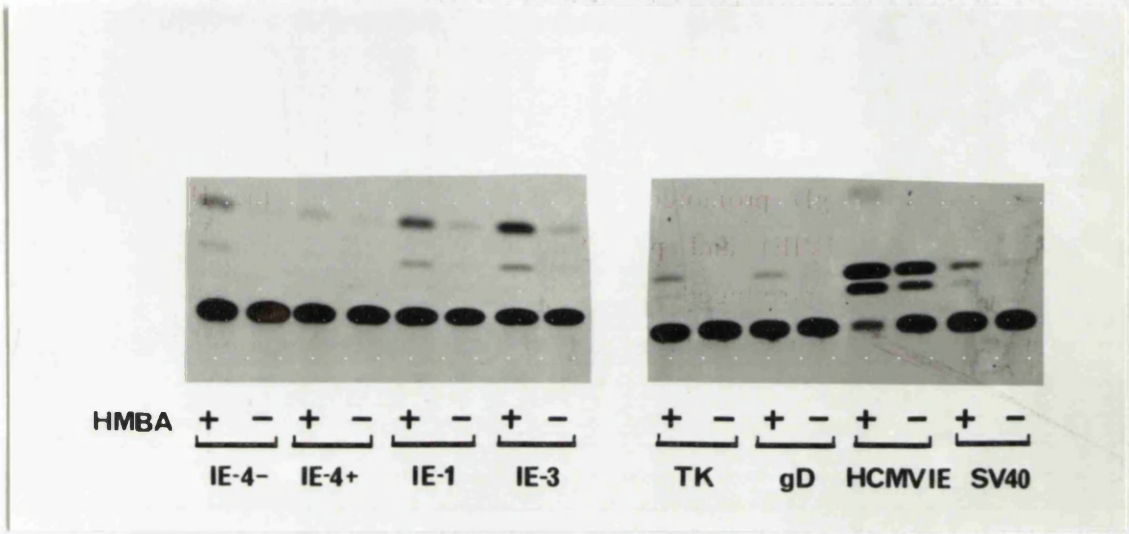
This experiment has been repeated, and the results obtained here confirmed.

The radioactivity in the substrate and the acetylated product spots was determined by scintillation counting to calculate the percent conversion to product.

	cpm -HMBA		%	cpm + HMBA		%	fold increase
	substrate	product	conversion	substrate	product	conversion	CAT activity + HMBA
PROMOTER							
IE4⁻	83857	655	0.78%	58635	2359	3.87%	4.96
IE4⁺	71559	620	0.86%	82503	1038	1.24%	1.44
IE1	100607	1190	1.17%	81507	4607	5.35%	4.57
IE3	78219	1355	1.7%	82939	7809	8.61%	5.06
TK	83302	238	0.28%	68610	940	1.35%	4.82
gD	61762	285	0.46%	56148	637	1.12%	2.43
HCMV IE	39742	20606	34.15%	2352	79640	97.13%	2.84*
SV40	71782	348	0.48%	72139	1420	1.9%	3.96

* The value for HCMV IE is likely to have been underestimated, as the assay was not in the linear part of the reaction.

Figure 4.7



induction of CAT activity may act at step intimately involved in transcription initiation (Section 4.5).

4.3.2 Effect of HMBA on a range of HSV-1 mutants

The question of specificity was subsequently addressed using a range of different HSV-1 mutant viruses, and testing whether HMBA could complement their defects in plaque assays. In contrast with the previous plasmid based assay (Section 4.3.1), the use of mutant viruses allows direct investigation into the effect of HMBA on different promoters in the environment of the viral genome.

Previous work demonstrated that plaque formation by *in*1814 was increased by up to 10-fold when Vmw110 was provided *in trans* by plasmid transfection (Ace *et al.*, 1989), suggesting that the decreased level of Vmw110 contributed to the phenotype of *in*1814. This conclusion is supported by the fact that *dl*1403, an HSV-1 mutant in which the Vmw110 gene is deleted, shows a m.o.i-dependent growth defect in tissue culture cells similar to that of *in*1814 (Stow & Stow, 1986, 1989; Everett, 1989). To investigate whether the addition of HMBA could compensate for the absence of Vmw110, its effect on *dl*1403 was examined, by titration in the presence of 0, 1, 3 and 5mM HMBA (Table 4.3). Despite a sevenfold increase in titre being observed, the effects were not comparable to that obtained with *in*1814. Another mutant, *in*1825, in which the Vmw110 promoter is replaced by the Moloney Murine Leukemia virus enhancer and promoter, an element that is not utilized efficiently in HFL cells (C.M. Preston, unpublished results), was examined. This virus effectively behaves like *dl*1403 in HFL cells, and again, HMBA resulted in a limited (fourfold) increases in the titre of *in*1825 (Table 4.3). These results demonstrate that HMBA does not substantially compensate for the absence of Vmw110. The small increase in the titre of *dl*1403 and *in*1825 in the presence of HMBA was shown, by dot blot analysis of cytoplasmic RNA extracted with and without HMBA treatment, to be attributable to HMBA increasing the expression of other IE genes and hence partially overcoming the need for Vmw110 transactivation (C.M. Preston & J.I. Daksis, unpublished results).

Mutant *in*1820 has the Vmw110 promoter replaced by the Moloney murine leukemia virus enhancer in the *in*1814 genome. The presence of HMBA in the culture medium did not dramatically increase the titre of *in*1820 (Table 4.3). Neither *in*1825 nor *in*1820 produced any detectable Vmw110 RNA under IE conditions and exposure to HMBA did not switch on the expression of this gene. Dot blot analysis of cytoplasmic RNA reveals however, that there

Table 4.3 Effect of HMBA on the titre of *dl*1403 and *in*1825.

HMBA (mM)	Titre (pfu/ml) *dl*1403	*in*1825
0	5.0×10^6	3.2×10^7
1	3.4×10^7	3.6×10^7
3	3.4×10^7	8.7×10^7
5	2.6×10^7	1.3×10^8

HFL cells were infected with either *dl*1403 or *in*1825 and various concentrations of HMBA were added to the culture medium after the 1h adsorption and penetration period. Monolayers were fixed and stained two days post-infection and the resulting titres determined.

was an increase in the other IE RNA levels in the presence of 5mM HMBA (C.M. Preston & J.I. Daksis, unpublished results). HMBA can therefore act to increase the level of expression of the Vmw110 gene from its endogenous promoter but cannot detectably switch on its expression when under the control of the Moloney murine leukemia virus enhancer.

The responsiveness to HMBA of different promoter elements was investigated using mutant *in*1827, which has the Vmw110 promoter replaced by the major promoter/HCMV/enhancer in the *in*1814 background, and *in*1833, which has the SV40 promoter/enhancer driving β-galactosidase inserted into the TK gene, also in the *in*1814 background. Analysis of IE RNA accumulation in the presence of 5mM HMBA (Figure 4.8) demonstrated that the HCMV enhancer is responsive to activation by HMBA in this environment. In addition, despite the level of expression being extremely low, an increase in β-galactosidase RNA was also detected by dot blot analysis of cytoplasmic RNA in the presence of HMBA (Figure 4.8). Therefore, both the HCMV enhancer and the SV40 promoter/enhancer are responsive to HMBA when in the viral genome.

Mutant *in*1411 contains an insertion in the gene encoding Vmw175, such that no functional Vmw175 is produced, and consequently can only be grown on a complementing cell line. Exposure to HMBA did not allow *in*1411 to form plaques on HFL or BHK cells (results not shown), therefore HMBA does not act as or induce an active cellular homologue of Vmw175. Another mutant HSV-140, in which the HSV-1 Vmw175 gene is replaced by the VZV homologue 140,000 protein, may be propagated, but fails to form plaques on BHK cells. In contrast, *in*1411, can only be propagated on a complementing cell line expressing Vmw175 (Disney & Everett, 1990).

Exposure to HMBA had no effect on the ability of HSV-140 to form plaques on HFL or BHK cells (results not shown), thus confirming the conclusions from the titration experiments with *in*1411, that HMBA does not act as or induce a homologue of Vmw175.

Figure 4.8 Effect of HMBA on IE RNA accumulation during *in*1827 and *in*1833 infection

HFL cells were infected with 100 particles of *in*1814 (14), 1814R (R), *in*1827 (27), *in*1833 (33) per cell, or mock-infected (M), in the presence of 25μg/ml cycloheximide. After adsorption and penetration, culture medium containing cycloheximide and, where appropriate, 5mM HMBA was added. After a further 4h, RNA was extracted and 3, 1 or 0.3μg was applied to nitrocellulose membranes. Membranes were probed with radiolabelled DNA fragments specific for IE genes 1 (encodes Vmw110), 2 (encodes Vmw63), 3 (encodes Vmw175) or 4 (encodes Vmw68), or mouse γ-actin (ACTIN). The *in*1833 RNA was also probed with a radiolabelled fragment specific for β-galactosidase.

This experiment has been repeated, and the results obtained here confirmed.

The absorbance at 260nm was measured to determine the concentration of the RNA.

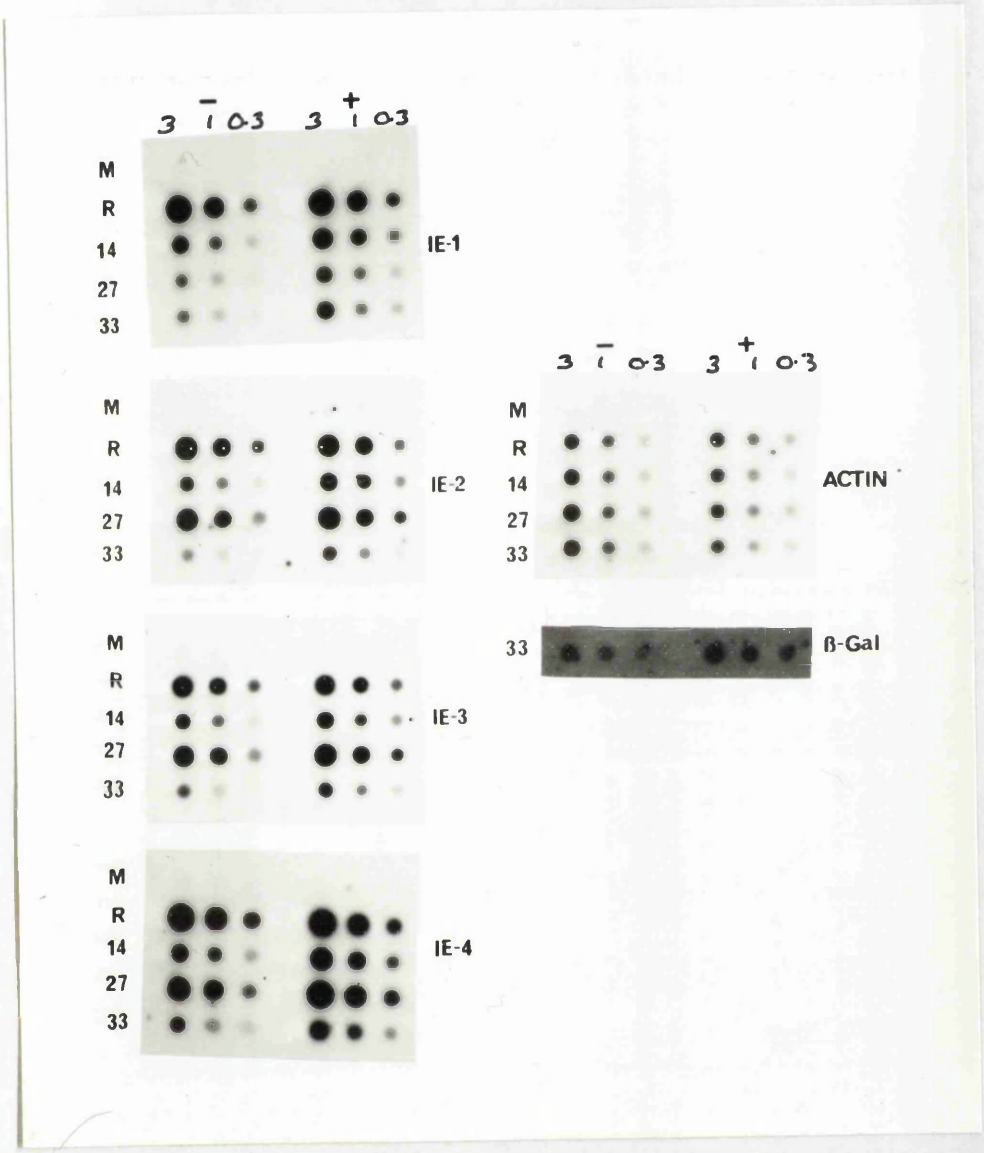
Hybridisation signals in figure 4.8 were quantified by densitometric measurement of spot intensities. The percent area under the curves after gaussian integration analysis are the values noted.

	-HMBA			+HMBA						
	3.0	1.0	0.3	3.0	1.0	0.3μg RNA	Mean Fold Increase			MeanIncrease
1814R										
IE1	20.1	15.7	8.6	22.4	22.6	10.6	1.11	1.44	1.2	1.25
IE2	28.7	17.4	2.7	30.6	18.0	2.5	1.07	1.03	0.93	1.01
IE3	17.3	21.0	7.9	28.7	18.2	6.9	1.66	0.87	0.87	1.13
IE4	28.7	14.0	3.1	33.0	16.1	5.2	1.15	1.15	1.68	1.33

	-HMBA			+HMBA						
	3.0	1.0	0.3	3.0	1.0	0.3μg RNA	Mean Fold Increase			MeanIncrease
***in*1814**										
IE1	23.7	10.4	2.0	34.3	21.3	8.4	1.45	2.05	4.2	2.57
IE2	21.3	10.3	2.9	34.5	24.2	6.8	1.62	2.35	2.34	2.1
IE3	27.2	12.0	2.2	35.2	16.4	7.0	1.29	1.37	3.18	1.95
IE4	22.3	12.5	3.8	33.2	20.1	8.2	1.49	1.61	2.16	1.75

	-HMBA			+HMBA						
	3.0	1.0	0.3	3.0	1.0	0.3μg RNA	Mean Fold Increase			MeanIncrease
in*1827										
IE1	19.7	6.9	1.5	45.3	19.8	6.8	2.3	2.87	4.5	3.2
IE2	23.7	15.9	5.0	27.3	19.1	9.1	1.15	1.2	1.82	1.39
IE3	23.6	12.0	4.0	40.4	15.3	4.6	1.71	1.28	1.15	1.38
IE4	17.3	21.2	6.9	21.8	24.8	8.1	1.26	1.43	1.17	1.29

Figure 4.8



	-HMBA			+HMBA			Mean Fold Increase			MeanIncrease
	3.0	1.0	0.3	3.0	1.0	0.3µg RNA				
***in*1833**										
IE1	17.5	5.1	0.8	53.9	16.1	6.6	3.08	3.16	8.25	4.83
IE2	17.8	5.4	2.3	48.3	21.5	4.7	2.71	3.98	2.04	2.91
IE3	17.0	4.4		26.0	6.1		1.53	1.39		1.46
IE4	21.4	8.3	1.1	42.7	20.1	6.5	1.99	2.4	5.9	3.43
Bgal	18.7	10.2	7.7	31.5	20.2	11.8	1.68	1.98	1.53	1.73

	-HMBA			+HMBA			Mean Fold Increase			MeanIncrease
	3.0	1.0	0.3	3.0	1.0	0.3µg RNA				
ACTIN										
1814R	26.1	15.8	6.0	31.9	13.7	6.5	1.22	0.87	1.08	1.06
*in*1814	25.9	24.0	7.2	29.2	9.7	3.9	1.13	0.61	0.54	0.76
*in*1827		26.9	9.5		15.3	5.9		0.57	0.62	0.6
*in*1833	39.8	18.2	10.9	22.0	8.0	1.0	0.55	0.44	0.1	0.36

* The value for *in*1827 may be biased due to the obvious underestimation of the titre, causing an unexpectedly high accumulation of IE2, 3 and 4 mRNAs compared to *in*1814.

4.4 Effect of HMBA on protein/DNA complex formation

4.4.1 HMBA does not promote IEC formation

As HMBA complements the effects of the Vmw65 mutation in *in*1814, it was considered possible that HMBA may act as or induce a cellular homologue of Vmw65, and a number of possible mechanisms by which this may occur can be envisaged. For example, HMBA may activate a specific cellular promoter and switch on the synthesis of a cellular homologue of Vmw65. However, the results from the dot blot analysis (Figure 4.5) demonstrate that the HMBA effect can occur in the presence of cycloheximide. In addition, the pretreatment of HFL cells with HMBA prior to infection with *in*1814 had no effect on the titre, suggesting that HMBA may transiently modify a protein already present. It is possible that HMBA activates a pre-existing cellular homologue of Vmw65, perhaps through a post-translational modification such as phosphorylation. Alternatively, HMBA may act as as physical 'linker' molecule facilitating an interaction between the mutant Vmw65 and Oct-1. Finally, HMBA itself may, because of its negative charge, form a complex with Oct-1 at the TAATGARAT and activate transcription. A number of gel retardation assays were performed to investigate these possibilities.

Nuclear extracts were prepared from untreated HeLa cells and from HeLa cells exposed to HMBA for 3h. Gel retardation assays were carried out to compare complex formation between untreated and HMBA-treated nuclear extracts with oligonucleotides containing the Ig octamer (01/02), and modified versions of the Ig octamer containing GCGGTAATGAGAT (JD1/JD2) and ATGCTAATGAGATAT (027/028), in the presence or absence of virion extract containing partially purified Vmw65 (Figure 4.9). The inclusion of HMBA-treated nuclear extract did not induce IEC formation in the absence of Vmw65 (lanes 3, 4, 7, 8, 11, 12), and did not affect the ability of the extract to form the IEC in conjunction with wild type virion extract (lanes 1, 2, 5, 6, 9, 10).

No novel complexes were detected using HMBA-treated nuclear extracts in conjunction with 01/02 and JD1/JD2 (Figure 4.9). However, in the absence of virion extract, a shifted complex of slightly greater mobility than the IEC was detected with the 027/028 oligonucleotide using both untreated and HMBA-treated extracts (Figure 4.9, lanes 11, 12).

Figure 4.9 Analysis of protein/DNA complex formation at TAATGARAT and octamer-containing DNA fragments

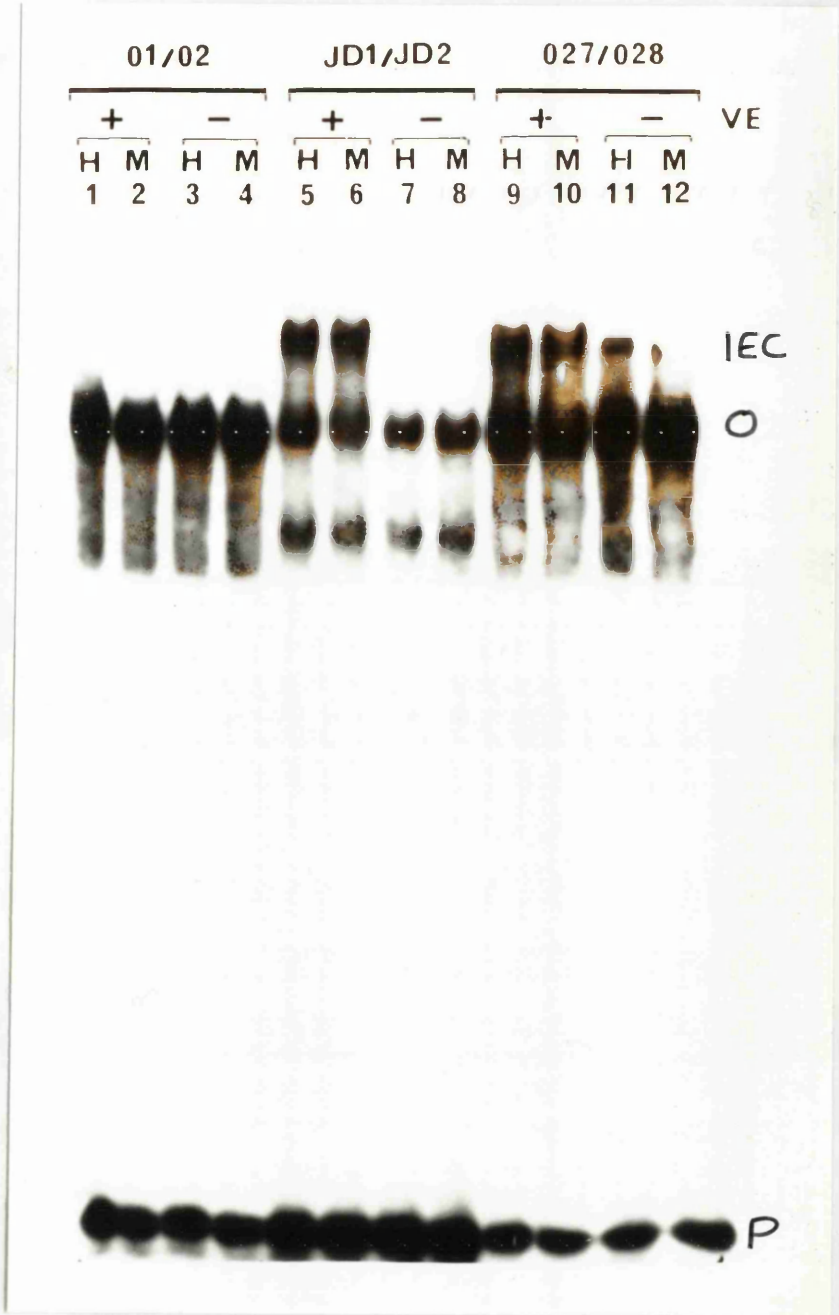
Comparison of complex formation using 5µg nuclear extract prepared from mock (M) or HMBA-treated (H) HeLa cells, in the presence (+) or absence (-) of wild type virion extract on oligonucleotides 01/02 (lanes 1-4), JD1/JD2 (lanes 5-8) and 027/028 (lanes 9-12). The sequences of the oligonucleotides used in this assay are listed in section 2.5.

The immediate early complex, containing Vmw65 and Oct-1, is denoted by IEC.

O represents the complex between DNA and Oct-1. The unbound labelled oligonucleotide is denoted by P. The origin of the additional complexes is unknown, but may be due to proteolysis.

Before virion extracts were analyzed in gel retardation assays, protein level comparisons were made by SDS-PAGE and coomassie brilliant blue staining.

Figure 4.9


01/02
JD1/JD2
027/028
+
−
+
−
+
−
VE
H M H M H M H M H M H M
1 2 3 4 5 6 7 8 9 10 11 12
IEC
O
P

The composition of the complex denoted X (Figure 4*B*) is unknown. However, it was noted that an increase in complex X occurred with the addition of nonradioactive 027/028 oligonucleotide, containing a strong Oct-1 binding site (Figure 4*D*, lanes 2-5), when compared to lane 1.

Gel retardation assays were carried out to determine whether the IEC could be detected in conjunction with *in*1814 virion extract on oligonucleotides JD1/JD2 and 027/028 using untreated and HMBA-treated HeLa cell nuclear extracts.

To determine whether the shifted band obtained with the 027/028 oligonucleotide is Oct-1 specific, competition assays were performed using unlabelled oligonucleotides containing a strong Oct-1 binding site (01/02), CCAAT box (CC1/CC2) and Ad MLP (L1/L2). Binding assays were performed using 5µg untreated HeLa nuclear extract in conjunction with 0, 6, 20, 60 and 100-fold excess of unlabelled 01/02 oligonucleotide, 6, 20 and 100-fold excess of unlabelled L1/L2 and 6, 20 and 100-fold excess of unlabelled CC1/CC2 for 15min at 25°C. Radiolabelled 027/028 probe was subsequently added and the reactions incubated for a further 30min (Figure 4.10). The addition of increasing concentrations of unlabelled 01/02, containing a sequence which constitutes a strong Oct-1 binding site, namely the Ig octamer, competed for the formation of the upper complex (Figure 4.10, lanes 1-6). In contrast, addition of 6, 20 and 100-fold excess of oligonucleotides representing the Ad MLP (Figure 4.10, lanes 7, 8, 9) and the CCAAT box (Figure 4.10, lanes 10, 11, 12) did not interfere with the formation of the upper complex. The shifted complex detected with the 027/028 oligonucleotide is therefore Oct-1 specific, as it was specifically competed using an unlabelled Oct-1 binding site. The 027/028 oligonucleotide also contains a second conserved element, the heptamer sequence (CTCATGA) which is recognized by both Oct-1 and Oct-2 (Verrijzer *et al.*, 1992). Therefore, the 027/028 oligonucleotide has the potential to bind two Oct-1 molecules, one at a strong binding site and another at this weaker heptamer site. This hypothesis has recently been confirmed using DNaseI footprinting analysis (C.M. Preston, unpublished results).

As pretreatment of HFL cells does not have any effect on the subsequent titre of *in*1814, it is possible that HMBA-induced changes could not be detected in gel retardation assays using HMBA-treated extracts. Therefore, *in*1814- and 1814R-infected HeLa cell nuclear extracts were prepared in the presence and absence of HMBA. HeLa cells were infected at 100 particles per cell of *in*1814 or 1814R, in the presence of 50µg/ml cycloheximide, with or without 5mM HMBA, and nuclear extracts were prepared after 4h. Figure 4.11 demonstrates that IEC formation was only observed using 1814R-infected extracts (lanes 4 and 5), not with *in*1814-infected extracts, even with HMBA treatment (lanes 2 and 3). These results are in agreement with those of Ace *et al.* (1989) who demonstrated that the use of *in*1814 virion extract and *in*1814-infected cell nuclear extracts did not overcome the inability to form the IEC. Figure 4.12 illustrates that HMBA-treated HeLa cell nuclear extract does not complement the Vmw65 mutation in *in*1814 as it does not promote IEC formation (Figure 4.12, lanes 2 and 4).

Figure 4.10 Competition analysis of complex formation with 027/028 oligonucleotide

Gel retardation assays performed using 5µg untreated HeLa nuclear extract, in conjunction with radiolabelld 027/028 oligonucleotide and 0 (lane 1), 6 (lane 2), 20 (lane 3), 60 (lane 4) and 100-fold (lane 5) excess of non-radioactive 027/028, containing a strong Oct-1 binding site, 6 (lane 6), 20 (lane 7) and 60-fold (lane 8) excess L1/L2 containing the Ad MLP and 6 (lane 9), 20 (lane 10) and 60-fold (lane 11) excess CC1/CC2, containing the CCAAT box. The additional shifted complex is denoted by the arrow. The sequences of the oligonucleotides used in this assay are listed in section 2.5.

The immediate early complex, containing Vmw65 and Oct-1, is denoted by IEC.

O represents the complex between DNA and Oct-1. The unbound labelled oligonucleotide is denoted by P. The origin of the additional complexes is unknown, but may be due to proteolysis. The composition of complex X is unknown.

Before virion extracts were analyzed in gel retardation assays, protein level comparisons were made by SDS-PAGE and coomassie brilliant blue staining.

Figure 4.10

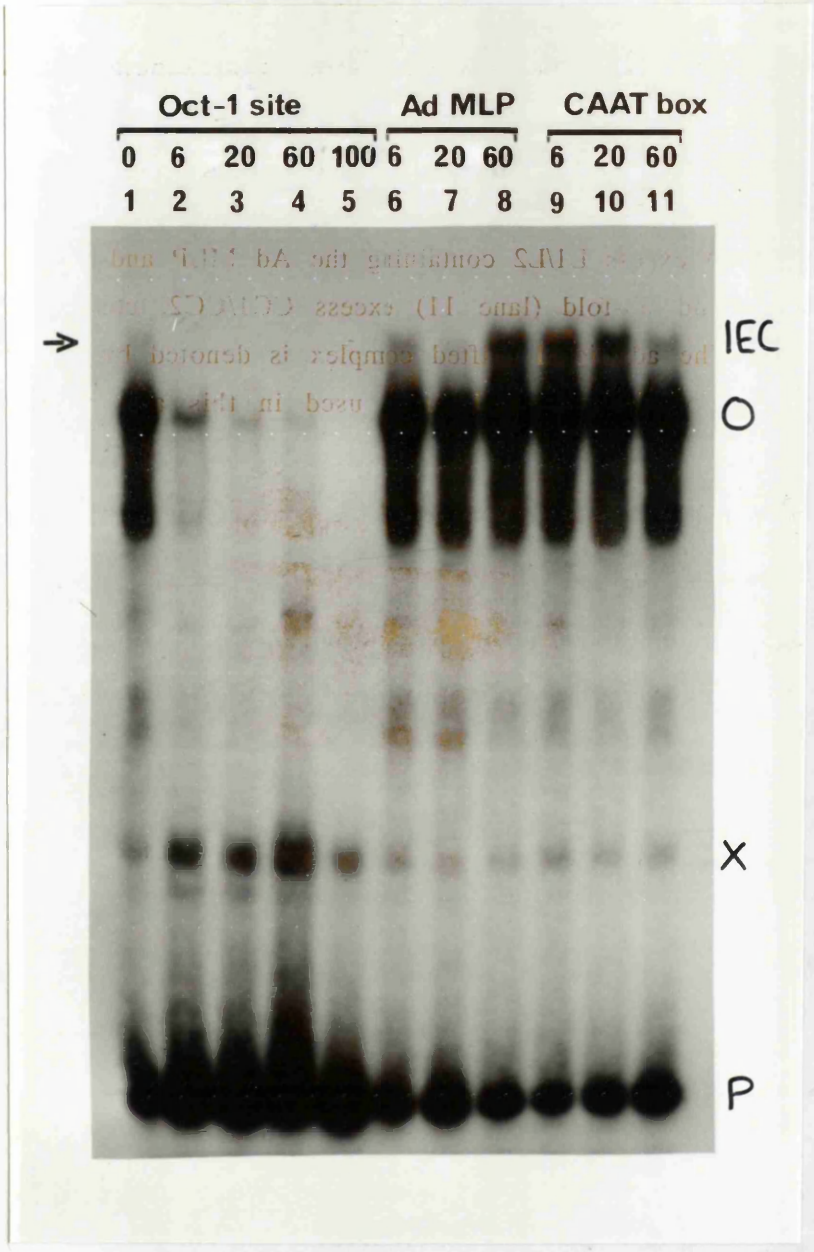

Oct-1 site
Ad MLP
CAAT box
0 6 20 60 100 6 20 60 6 20 60
1 2 3 4 5 6 7 8 9 10 11
IEC
O
X
P

Figure 4.11 Analysis of complex formation with untreated and HMBA-treated infected cell extracts

Comparison of complex formation using 5μg mock-treated (M, lanes 1, 3, 4) or HMBA-treated (H, lanes 3 and 5) *in*1814- (14, lanes 2 and 3) or 1814R-infected (R, lanes 4 and 5) HeLa cell nuclear extracts with the JD1/JD2 oligonucleotide containing the TAATGARAT element. The sequences of the oligonucleotides used in this assay are listed in section 2.5.

The immediate early complex, containing Vmw65 and Oct-1, is denoted by IEC.

O represents the complex between DNA and Oct-1. The unbound labelled oligonucleotide is denoted by P. The origin of the additional complexes is unknown, but may be due to proteolysis.
Before virion extracts were analyzed in gel retardation assays, protein level comparisons were made by SDS-PAGE and coomassie brilliant blue staining.

Figure 4.11

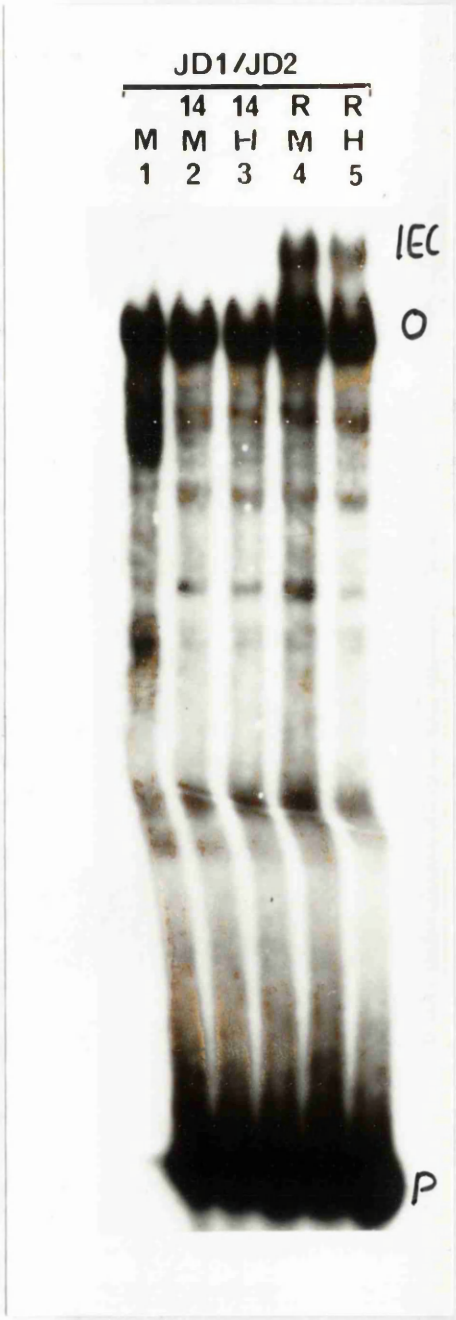
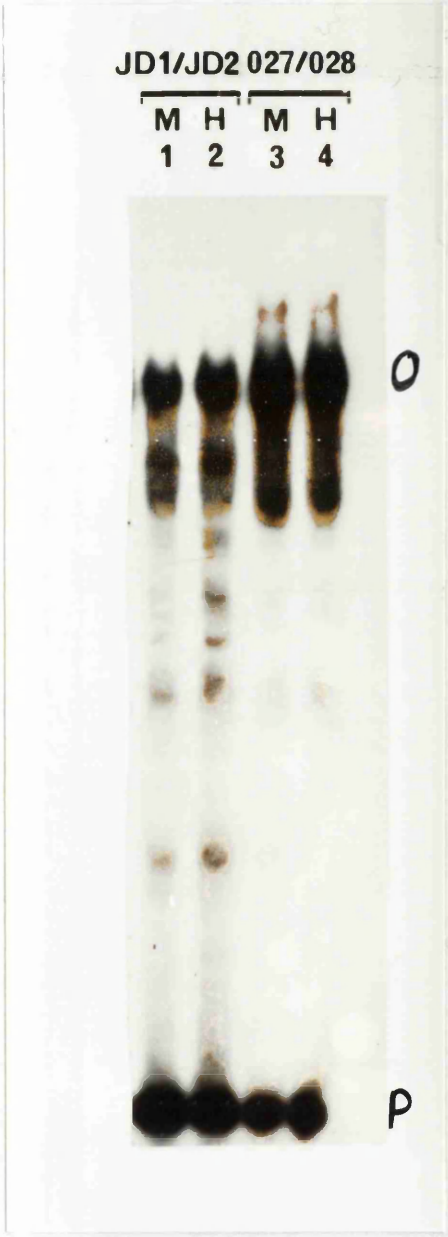


Figure 4.12 Analysis of complex formation with untreated and HMBA-treated extracts in conjunction with *in*1814 virion extract

Comparison of complex formation using 5µg mock (M) or HMBA-treated (H) HeLa cell nuclear extract, in the presence of *in*1814 virion extract, on oligonucleotides JD1/JD2 (lanes 1 and 2) and 027/028 (lanes 3 and 4). The sequences of the oligonucleotides used in this assay are listed in section 2.5.

O represents the complex between DNA and Oct-1. The unbound labelled oligonucleotide is denoted by P. The origin of the additional complexes is unknown, but may be due to proteolysis.
Before virion extracts were analyzed in gel retardation assays, protein level comparisons were made by SDS-PAGE and coomassie brilliant blue staining.

Figure 4.12



To determine whether the HMBA molecule itself forms a physical link between the mutant Vmw65 and Oct-1, gel retardation assays were performed with or without the inclusion of 5mM HMBA in the binding reactions, using mock, *in*1814- and 1814R-infected HeLa cell nuclear extracts. The nuclear extracts tested were either untreated or exposed to HMBA prior to the preparation of the extract (Figure 4.13). The inclusion of 5mM HMBA in the binding assays did not promote IEC formation at the TAATGARAT element in the presence of the mutant Vmw65 (Figure 4.13, lanes 9 and 10), thus it can be concluded that HMBA does not appear to act as a linker molecule aiding the binding of the mutant Vmw65 to Oct-1 to form the IEC.

The results from the gel retardation assays demonstrate that HMBA neither acts as, activates nor induces a cellular homologue of Vmw65. HMBA must therefore function through an alternative mechanism, that does not require IEC formation, where it can facilitate complementation of the phenotype of *in*1814.

4.4.2 Effect of HMBA treatment on complex formation at other promoter elements

As HMBA-treated HeLa cell nuclear extracts did not promote IEC formation in the absence of Vmw65, gel retardation assays were performed to determine whether the use of HMBA-treated HeLa cell nuclear extract promoted the binding of transcription factors at the Ad MLP or CCAAT box.

Binding assays were performed using 5µg HeLa cell nuclear extract, *in*1814- and 1814R-infected nuclear extracts, produced in the presence or absence of HMBA exposure. Figure 4.14 (lanes 1-5) reveals that HMBA-treatment of *in*1814- or 1814R-infected nuclear extracts did not increase complex formation at the CCAAT box, but appeared to interfere with complex formation. In addition figure 4.14 (lanes 13 and 14) demonstrates that HMBA-treated HeLa cell nuclear extract showed decreased complex formation at the CCAAT box compared to the untreated HeLa cell extract.

Similarly, analysis of complex formation at the Ad MLP using 5µg *in*1814- or 1814R-infected extracts, generated in the presence or absence of HMBA-treatment, revealed that HMBA-treatment does not promote complex formation at the Ad MLP (Figure 4.14, lanes 6-10). In fact, complex formation at the Ad MLP using HMBA-treated HeLa cell nuclear extract appeared to be slightly reduced in comparison with the untreated HeLa cell nuclear extract (Figure 4.14, lanes 11 and 12).

Figure 4.13 Analysis of complex formation in the presence and absence of 5mM HMBA

Gel retardation assays were performed to compare complex formation at the TAATGARAT motif using mock- (M, lanes 1, 2, 7 and 8), *in*1814- (14, lanes 3, 4, 9, 10) and 1814R-infected (R, lanes 5, 6, 11, 12) HeLa cell nuclear extracts, prepared with (H, lanes 2, 4, 6, 8, 10, 12) or without (lanes 1, 3, 5, 7, 9, 11) HMBA treatment. Binding assays were carried out in the presence (lanes 1-6) or absence (lanes 7-12) of 5mM HMBA in the reaction mix. The sequences of the oligonucleotides used in this assay are listed in section 2.5.

The immediate early complex, containing Vmw65 and Oct-1, is denoted by IEC.

O represents the complex between DNA and Oct-1. The unbound labelled oligonucleotide is denoted by P. The origin of the additional complexes is unknown, but may be due to proteolysis.

Before virion extracts were analyzed in gel retardation assays, protein level comparisons were made by SDS-PAGE and coomassie brilliant blue staining.

Figure 4.13

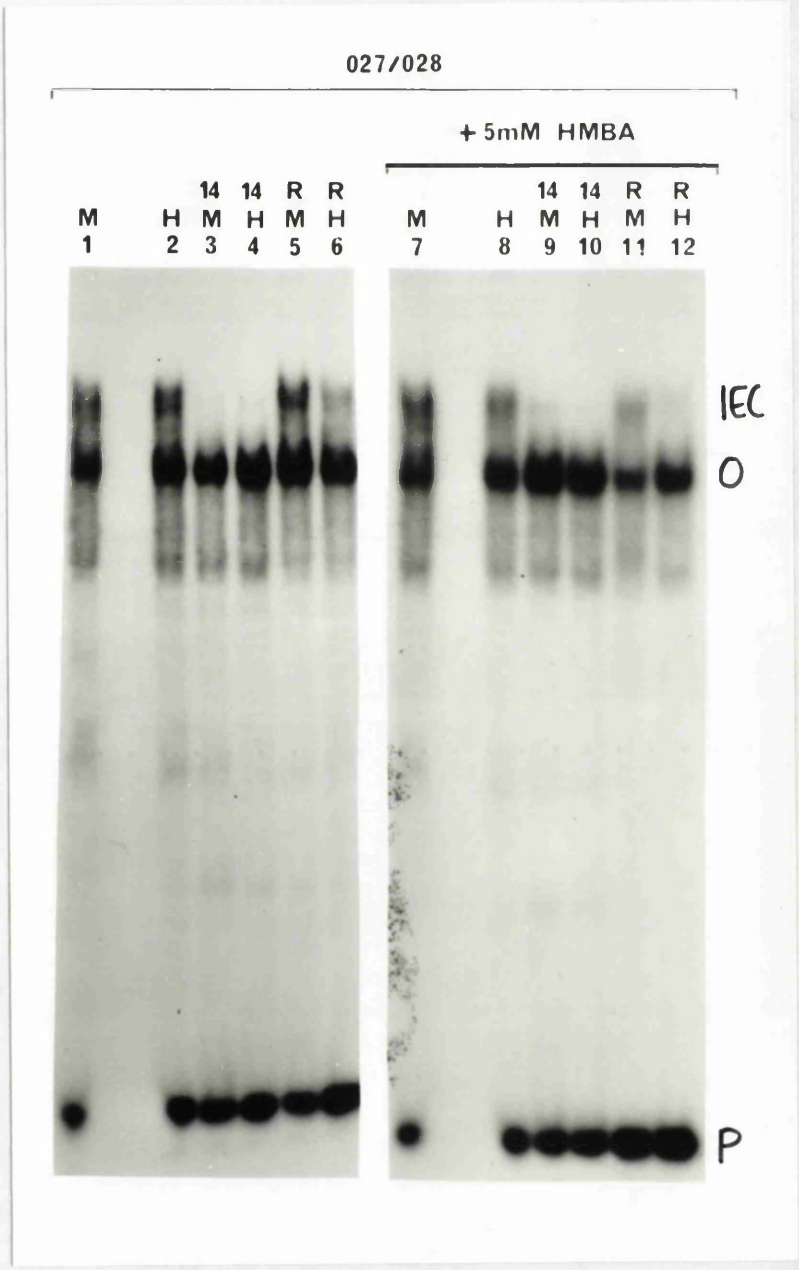
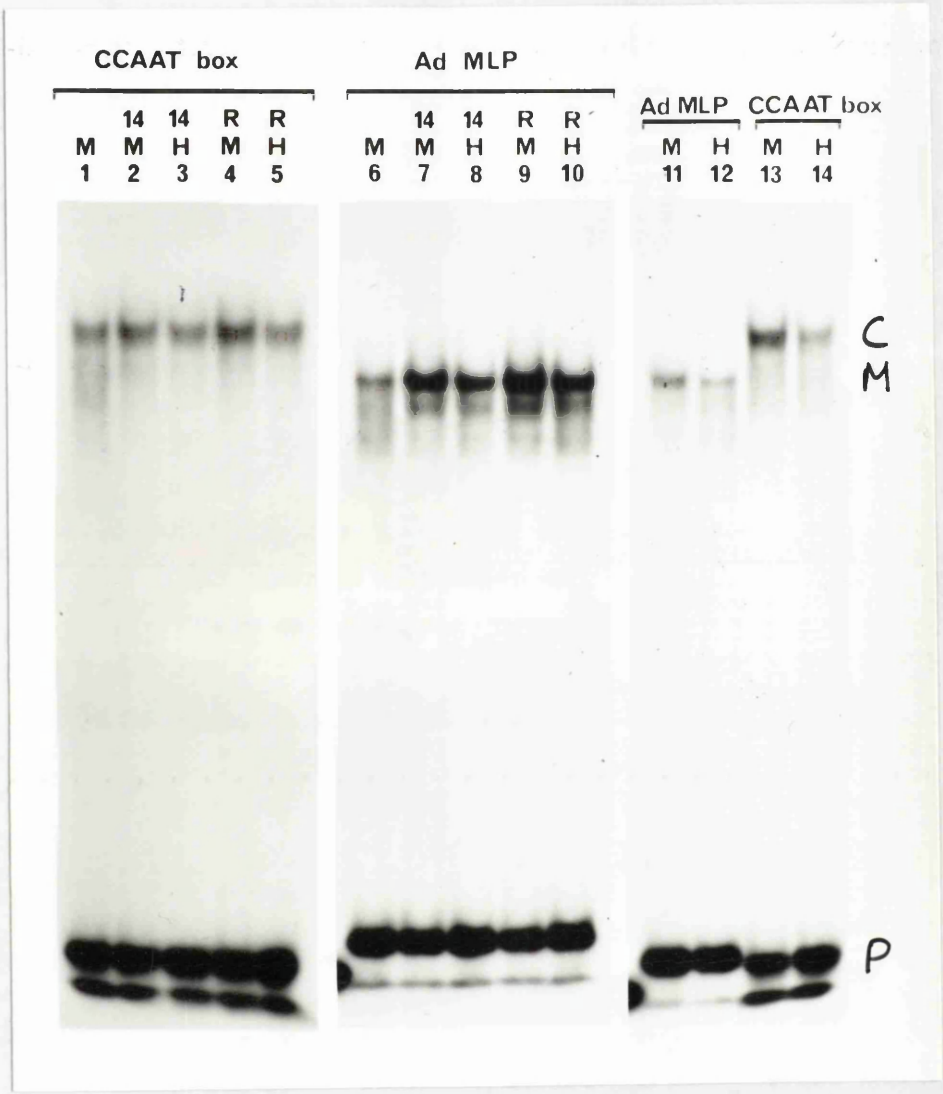

027/028
+ 5mM HMBA
M 1
H 2
14 M 3
14 H 4
R M 5
R H 6
M 7
H 8
14 M 9
14 H 10
R M 11
R H 12
IEC
O
P

Figure 4.14 Analysis of complex formation at the CCAAT box and Ad MLP

Gel retardation assays were performed to analyse complex formation at the CCAAT box (lanes 1-5) and AD MLP (lanes 6-10) using 5μg mock (M), *in*1814- (14, lanes 2, 3, 7, 8) and 1814R- infected (R, lanes 4, 5, 9, 10) HeLa cell nuclear extracts, prepared with (H, lanes 3, 5, 8, 10) or without (M, lanes 1, 2, 4, 6, 7, 9) HMBA treatment. The complexes formed using mock-infected (M, lanes 11 and 13) and HMBA-treated mock (H, lanes 12 and 14) extracts were also compared (lanes 11-14). The sequences of the oligonucleotides used in this assay are listed in section 2.5.

C represents the major complex formed on the CCAAT box oligonucleotide. M represents the major complex formed on the Ad MLP oligonucleotide. P represents the unbound oligonucleotide. The precise composition of the other complexes is unknown.

Figure 4.14


CCAAT box
Ad MLP
Ad MLP
CCAAT box
M 1
14 M 2
14 H 3
R M 4
R H 5
M 6
14 M 7
14 H 8
R M 9
R H 10
M 11
H 12
M 13
H 14
C
M
P

Therefore, HMBA-treatment of *in*1814-, 1814R- and mock-infected HeLa cell nuclear extracts, does not promote complex formation at either the CCAAT box or Ad MLP compared to the level of complex formation seen using untreated extracts. In fact, HMBA-treatment of HeLa cells prior to the preparation of nuclear extracts appears to be associated with the depletion of factors required for complex formation at these motifs.

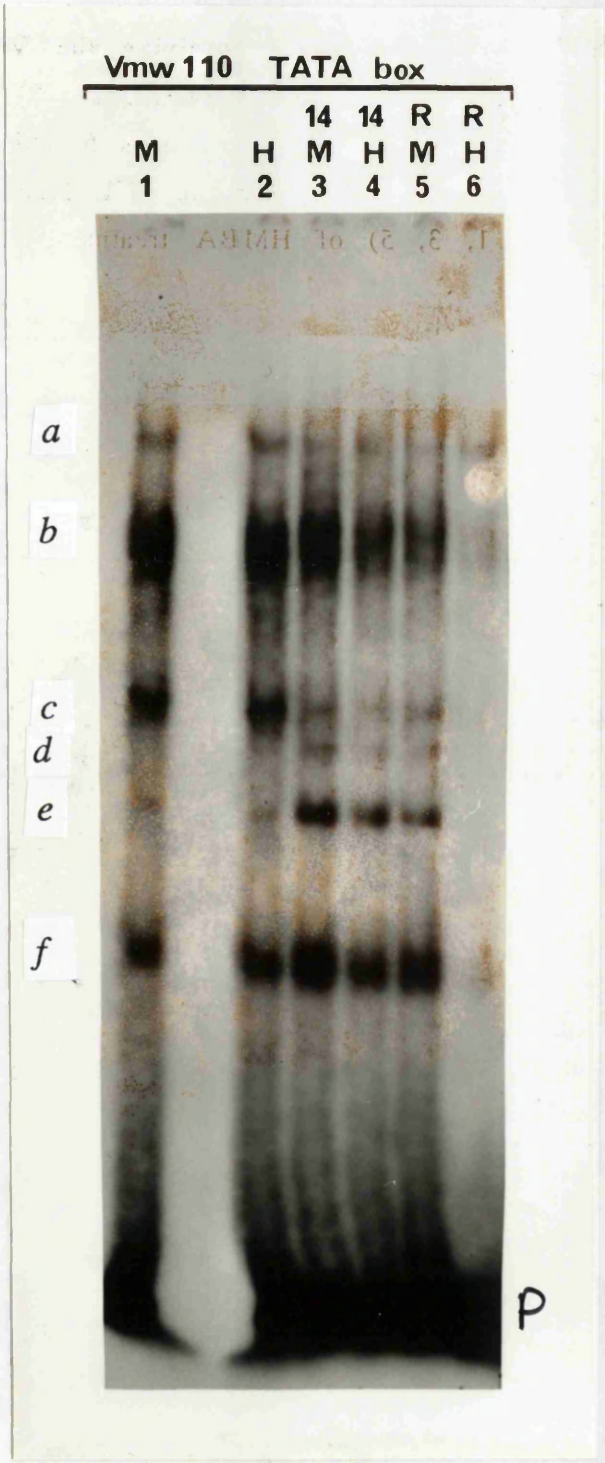
As HMBA-treatment does not increase complex formation at the TAATGARAT motif, the CCAAT box or Ad MLP, it was feasible to consider whether there are any affects on the formation of the protein-DNA complexes at the TATA box. An oligonucleotide representing the HSV-1 strain 17 Vmw110 TATA box was constructed and gel retardation assays were performed in conjunction with 5μg mock-, *in*1814- and 1814R-infected HeLa cell nuclear extracts, in the presence or absence of HMBA treatment (Figure 4.15). A number of different, as yet, unidentified complexes were detected using an oligonucleotide containing the Vmw110 TATA box. Although the results from the gel retardation assays suggest that HMBA-treatment does not increase complex formation at the TATA box, variations in the intensity of certain complexes was observed. The intensities of complexes *a*, *b* and *c* were greater using uninfected HeLa cell nuclear extract than with either *in*1814- or 1814R-infected HeLa cell nuclear extracts. This variation in complex formation between mock and infected HeLa cell nuclear extracts may reflect the sequestering of factors required for transcription initiation by the infecting viruses. In contrast, the intensity of complex *e* is greater using both *in*1814- and 1814R-infected HeLa nuclear extracts than with uninfected HeLa cell extract, while the formation of complex *f* appears to be unaltered by viral infection. A novel complex *d*, not detected using mock-infected HeLa cell nuclear extracts, is obtained using both *in*1814- and 1814R-infected HeLa cell extracts. HMBA treatment of *in*1814- and 1814R-infected HeLa cell nuclear extracts appears to slightly reduce the formation of complexes *b*, *c*, *d*, *e* and *f*. However, the significance of these observations, with respect to the effect of HMBA on the replication of *in*1814, have not yet been determined.

Figure 4.15 Analysis of complex formation at Vmw110 TATA box

Gel retardation assays were performed to analyse complex formation with an oligonucleotide containing the Vmw110 TATA box (Section 2.5) and 5µg mock- (M, lanes 1 and 2), *in*1814- (14, lanes 3 and 4) and 1814R-infected (R, lanes 5 and 6) HeLa cell nuclear extracts, prepared in the presence (H, lanes 2, 4, 6) or absence (M, lanes 1, 3, 5) of HMBA treatment. Complexes are denoted *a* to *f*.

P represents the unbound oligonucleotide.

Figure 4.15



4.5 Effect of HMBA during *in vitro* transcription

An *in vitro* transcription initiation system was employed using Manley whole cell HeLa extracts (Manley *et al.*, 1980), as a tool to investigate the exact mechanism whereby HMBA acts at the molecular level and reveal the signals involved in this process. The observation of Campbell *et al.* (1991), that sequences upstream of the TATA box may not be required for HMBA-mediated induction of CAT activity in transient transfection assays and the inability to demonstrate any sequence specificity (Section 4.3.1), raises the possibility that HMBA may act, in transient assays, at the level of transcription initiation. A feasible mechanism would involve the post-translational modification of a factor intimately involved in transcription initiation. The objective was therefore to prepare both untreated and HMBA-treated Manley whole cell HeLa extracts, and to determine the activity of each extract in an *in vitro* transcription reaction. If the HMBA-treated extracts showed a significant increase in the level of transcriptional activity when compared to the untreated extract, then the HMBA-treated extract would be fractionated. Subsequently, addition of the different fractions to the untreated extracts and determination of the resulting transcriptional activity would facilitate the isolation of the fraction, to reveal the component responsible for increased transcription. Diagrammatic representations of the plasmids used as templates for *in vitro* transcription reactions are depicted in appendix 1.

4.5.1 Effect of HMBA-treatment of cell extract on transcription by RNA polymerase II *in vitro*

In the first instance extract and DNA template concentrations were titrated to establish the optimum conditions for transcription reactions. Figure 4.16 illustrates the effect of different DNA template concentrations on the level of transcription. The plasmid construct, pML(C_2AT)$_{19}$, containing the Ad MLP (Sawadogo *et al.*, 1985) (Appendix 1) was cleaved with *Sma*I, and several concentrations of the resulting linear template were incubated with 150µg of untreated whole cell extract at 30°C for 2h. As Manley whole cell extracts contain relatively high concentrations of nucleic acids, mostly 18s and 28s RNA (Manley *et al.*, 1980), only 25% of the RNA produced was analysed to prevent overloading of the gel. The data presented in figure 4.16 (lane 3) demonstrates there is a critical DNA template concentration of 0.1µg for the

Figure 4.16 Titration of template DNA concentration

Titration of pML(C_2AT)$_{19}$ containing the Ad MLP template DNA (Section 2.4), cleaved with *Sma*I, to determine the optimum concentration for *in vitro* transcription. Reactions were carried out with 150μg untreated Manley whole cell HeLa extract and 0.05μg (lane 1), 0.07μg (lane 2), 0.1μg (lane 3) and 0.3μg (lanes 4 and 5) plasmid DNA. Lane 5 contains 1.0μg/ml α-amanatin. *In vitro* transcription reactions were carried out a 30° for 2h. Only 1/4 of each reaction mix was analysed on a denaturing 6% polyacrylamide/6M urea gel. The arrow denotes the 390 nucleotide transcript produced.

* See text, Section 4.5.1

Figure 4.16

* The bands denoted by B (Figure 4.16, lanes 4 and 5) only occur when the 390 nucleotide RNA transcript is detected. The origin and significance of this observation is unknown.

plasmid pML(C_2AT)$_{19}$ which had been linearized by *Sma*I using this particular extract, below which transcription is not detected. The inclusion of 1.0μg/ml α-amanatin in the reaction inhibits RNA polymerase II transcription, confirming that the band obtained is indeed a product of RNA polymerase II transcription (Figure 4.16, lane 5). The RNA transcript produced, denoted by the arrow (Figure 4.16) was, as expected, 390 nucleotides long (Appendix 1). The identity of the bands at the bottom of the gel (marked by *) is not known.

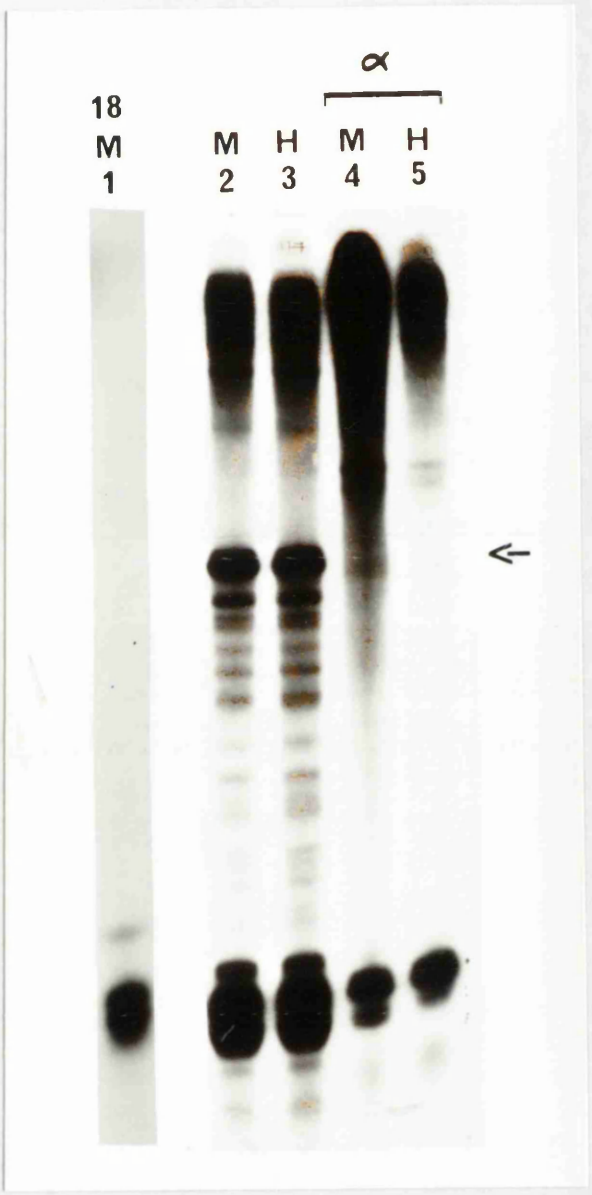
Each template used was titrated to determine the optimum concentration required for maximal activity in conjunction with a particular extract.

RNA dot blot analyses (Figure 4.5) revealed that exposure to HMBA during *in*1814 infection resulted in the greatest increase in Vmw110 RNA accumulation. Therefore *in vitro* transcription experiments were carried out to compare the activity of the Vmw110 promoter using untreated and HMBA treated Manley whole cell HeLa extracts. The plasmid pA7436, containing the regulatory sequences of the Vmw110 gene in a pUC18 vector, was cleaved with *Nde*I (Appendix 1) and incubations were carried out for 2h at 31°C using 0.1μg of this linear template with either untreated extract or extract which had been exposed to HMBA for 3h, in the presence or absence of 1.0μg/ml α-amanatin. To ensure that the additional smaller RNA products obtained (Figure 4.17, lanes 2-5) were not due to initiations occurring within the vector sequences, pUC18 was cleaved with *Nde*I, and 0.1μg of linearized template DNA was incubated with untreated extract as previously described. None of the RNA transcripts previously obtained after transcription of the template containing the Vmw110 promoter, were detected using the vector pUC18 as a template (Figure 4.17, lane 1). Therefore, the additional RNA products generated during transcription of the pA7436 template linearized by *Nde*I are not a consequence of transcription initiation occurring at pseudo TATA boxes within the vector sequences. Sawadogo and Roeder (1985) and Carcamo *et al.* (1989) proposed that a similar regular pattern of decreasing length transcripts, detected after *in vitro* transcription of a guanosine-free template, was due to reinitiation of transcription on the same template. When the transcription complex reaches the end of the linear template it does not fall off, preventing the next complex from generating a full length transcript. Thus a family of regularly decreasing-length transcripts is produced. It is therefore likely that the additional transcripts obtained from the Vmw110 promoter are a consequence of reinitiation of transcription on the same template.

Figure 4.17 Analysis of transcriptional activity of the Vmw110 promoter using untreated and HMBA-treated extracts

In vitro transcription reactions were carried out using 0.3μg pA736 template DNA containing the Vmw110 promoter (Section 2.4) cleaved with *Nde*I, with untreated (M, lanes 1, 2, 4) or HMBA-treated (H, lanes 3 and 5) Manley whole cell HeLa extracts in the presence (α, lanes 4 and 5) or absence (lanes 1, 2, 3) of 1.0μg/ml α-amanatin. 0.1μg *Nde*I pUC18 (18, lane 1) was incubated with untreared Manley whole cell HeLa extract. Reactions were incubated at 30° for 2h. The arrow denotes the 230 nucleotide transcript produced.

Figure 4.17



The difference in transcriptional activity between untreated and HMBA-treated Manley whole cell HeLa extracts was subsequently analysed from the HSV-1 Vmw68 promoter, in the presence (Figure 4.18, lanes 5 and 6) and absence (Figure 4.18, lanes 7 and 8) of upstream sequences containing the TAATGARAT motif, from the Ad MLP (Figure 4.18, lanes 1 and 2) and from the HCMV IE promoter (Figure 4.18, lanes 3 and 4). (See Appendix 1) Comparison of the amount of RNA products obtained from each of these promoters using untreated and HMBA-treated extracts, showed that HMBA-treatment of HeLa cells did not significantly increase the resulting transcriptional activity compared with the untreated extracts. There was neither a significant nor consistent difference in transcriptional activity between the untreated and HMBA-treated extracts to warrant fractionation of the HMBA-treated extract.

4.5.2 Does HMBA act directly on transcriptional activity?

As Vmw65 functions through its negatively charged activation domain (Triezenberg *et al.*, 1988, Cress & Triezenberg, 1991), it is feasible that the polar planar HMBA molecule is also active as a result of its overall negative charge. To investigate this possibility 0, 1, 3 and 5mM HMBA was added directly to a transcription reaction containing 0.1µg *Sma*I pML$(C_2AT)_{19}$, containing the Ad MLP, and 150µg of untreated extract (Figure 4.19). Analysis of the RNA produced revealed that the addition of HMBA had an inhibitory effect on transcription. Therefore, it is unlikely that HMBA functions simply as a consequence of its overall negative charge. Similar results were obtained using the HSV-1 Vmw110 promoter (results not shown).

4.5.3 Effect of HMBA-treatment of cell extract on RNA polymerase III transcription

In vitro transcription experiments were subsequently undertaken to determine whether HMBA-treatment of HeLa whole cell extracts has any effect on the level of transcription directed from an RNA polymerase III promoter. A circular template, containing the Ad VA promoter was transcribed using untreated and HMBA-treated extracts (Figure 4.20a). The inclusion of 1.0µg/ml α-amanatin in the reaction mix did not inhibit transcription (Figure 4.20a, lane 1), confirming that the RNA products obtained were produced by RNA polymerase III. The use of HMBA-treated extracts did not increase the level of VA RNA accumulation compared with untreated extract. The addition of increasing concentrations of HMBA to the

Figure 4.18 Analysis of transcriptional activity of from RNA polymerase II promoters using untreated and HMBA-treated extracts

In vitro transcription reactions were carried out using 150µg untreated (M, lanes 1, 3, 5, 7) or HMBA-treated (H, lanes 2, 4, 6, 8) Manley whole cell HeLa extracts with 0.3µg of pML(C_2AT)$_{19}$ containing the Ad MLP, cleaved with *Sma*I (lanes 1 and 2), pHCMV containing the HCMV IE promoter, cleaved with *Kas*I (lanes 3 and 4), pLWFU17 containing the IE-4 promoter plus additional upstream sequences, cleaved with *Eco*RI (lanes 5 and 6) or pLWP containing the IE-4 promoter, cleaved with *Eco*RI (lanes 7 and 8). Samples were incubated for 2h at 30°C. Plasmid constructs are described in detail in section 2.4.

Figure 4.18

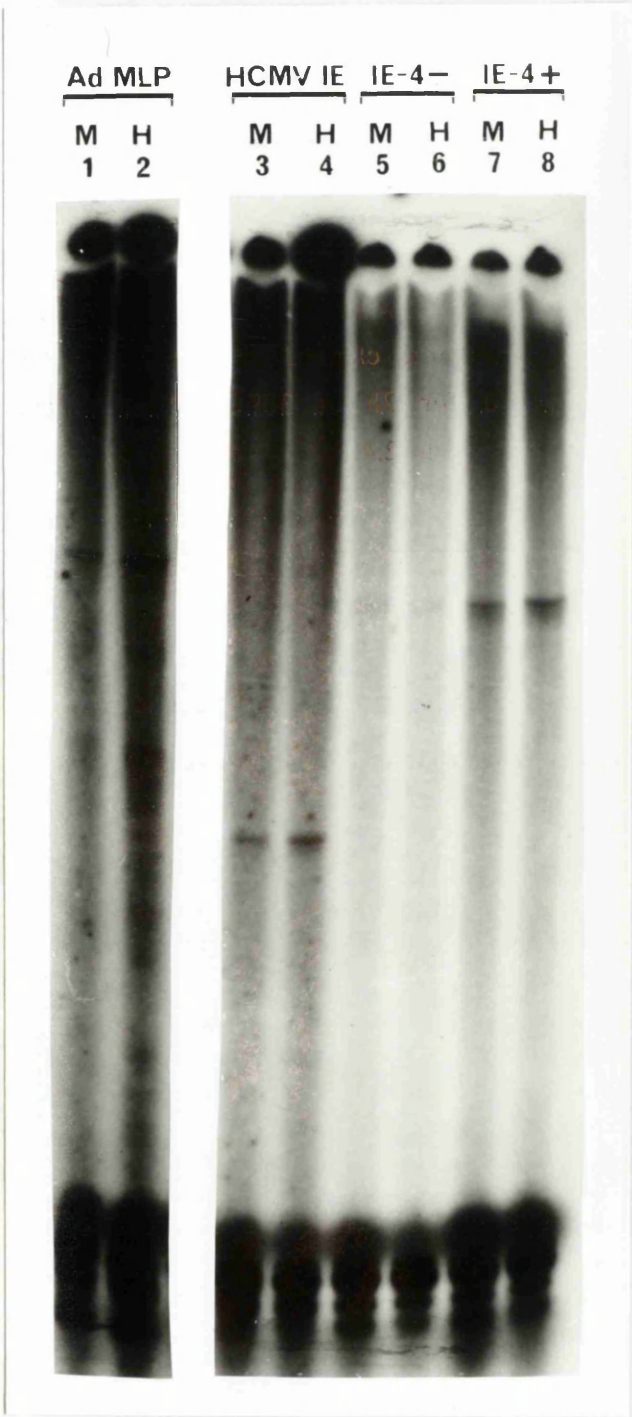


Figure 4.19 Effect of HMBA on transcriptional activity

0 (lane 1), 1 (lane 2), 3 (lane 3) and 5 (lane 4) mM HMBA was added directly to trancription reactions containing 150μg untreated Manley whole cell HeLa extract and 0.3μg pML$(C_2AT)_{19}$ (Section 2.4) containing the Ad MLP, cleaved with *Sma*I. The 390 nucleotide produced is denoted by the arrow.

Figure 4.19

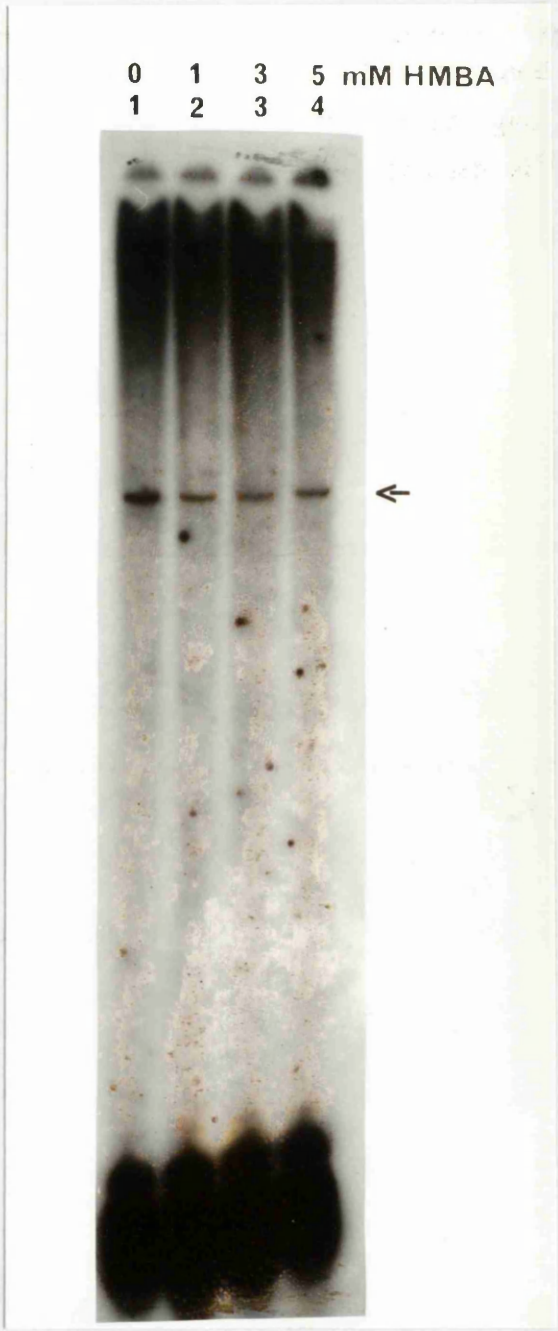
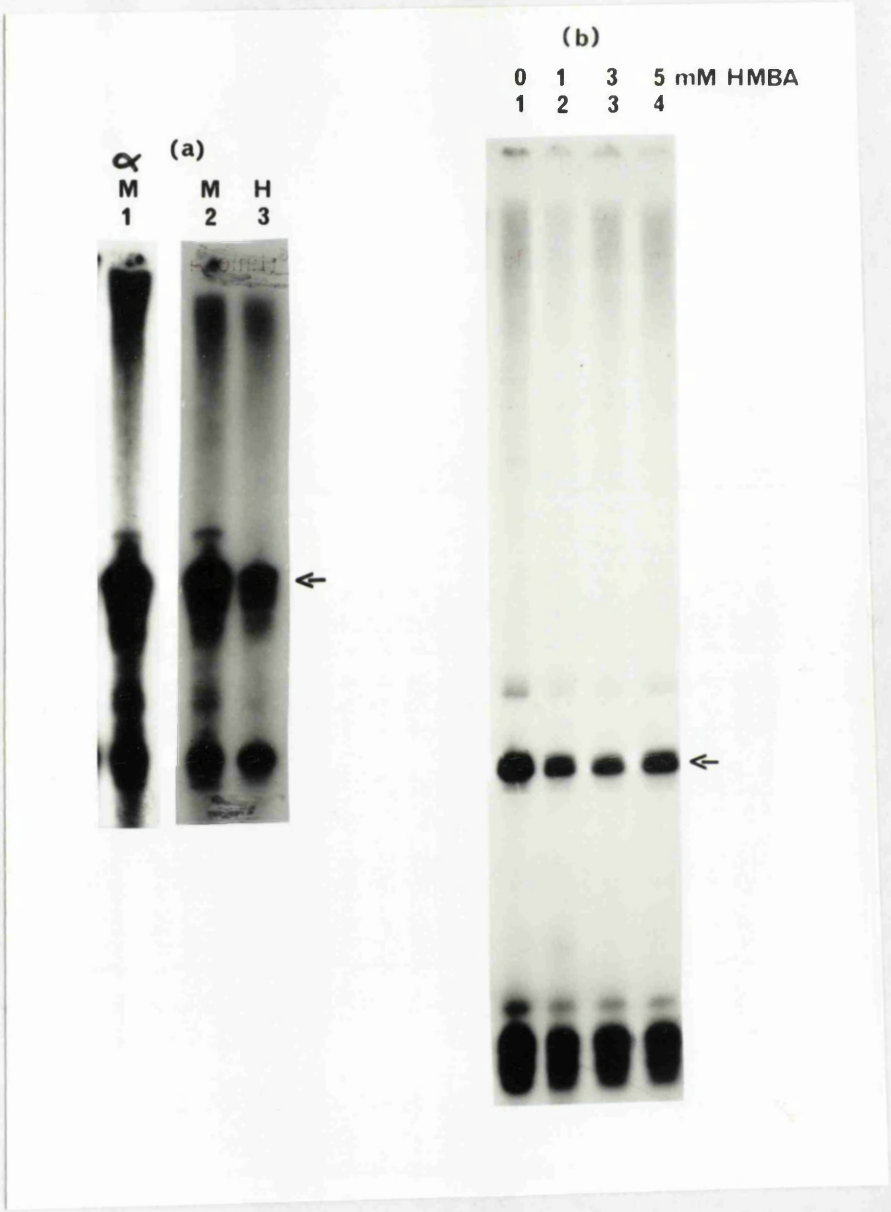


Figure 4.20 Analysis of RNA polymerase III transcription

(a) Comparison of transcription activity of untreated (M, lanes 1 and 2) and HMBA-treated (H, lane 3) Manley whole cell extract on the Ad VA RNA polymerase III transcribed promoter (Section 2.4). Lane 1 also contains 1.0μg/ml α-amanatin.

(b) Comparison of transcriptional activity of untreated Manley whole cell extract on the Ad VA promoter, in conjunction with 0 (lane 1), 1 (lane 2), 3 (lane 3) and 5mM (lane 4) HMBA.

Figure 4.20



transcription reaction also had an inhibitory effect on RNA polymerase III transcription from the Ad VA promoter (Figure 4.20b).

4.5.4 Effect of *in vitro* synthesized and purified Vmw65 and Oct-1 on transcription *in vitro*

Purified Vmw65 and the Oct-POU domain of Oct-1 (both kindly provided by Dr. B. Luisi), previously shown to form the IEC and bind to the octamer, respectively, when added to HeLa cell nuclear extract in gel retardation assays, were added to *in vitro* transcription reaction mixes containing templates with either the Vmw110 or Vmw63 promoters, to determine whether they were functionally active (Figure 4.21). No significant increases in the amount of RNA produced from either the Vmw110 or Vmw68 promoters were detected in the presence of either Oct-1 or Vmw65 singly or together. It is possible that the excess of Oct-1 and/or Vmw65 results in the "squelching" of additional cellular factors required for the formation of IEC.

4.6 Effect of HMBA on viral promoters in the cellular genome

4.6.1 Construction of TK$^+$-transformed cell lines

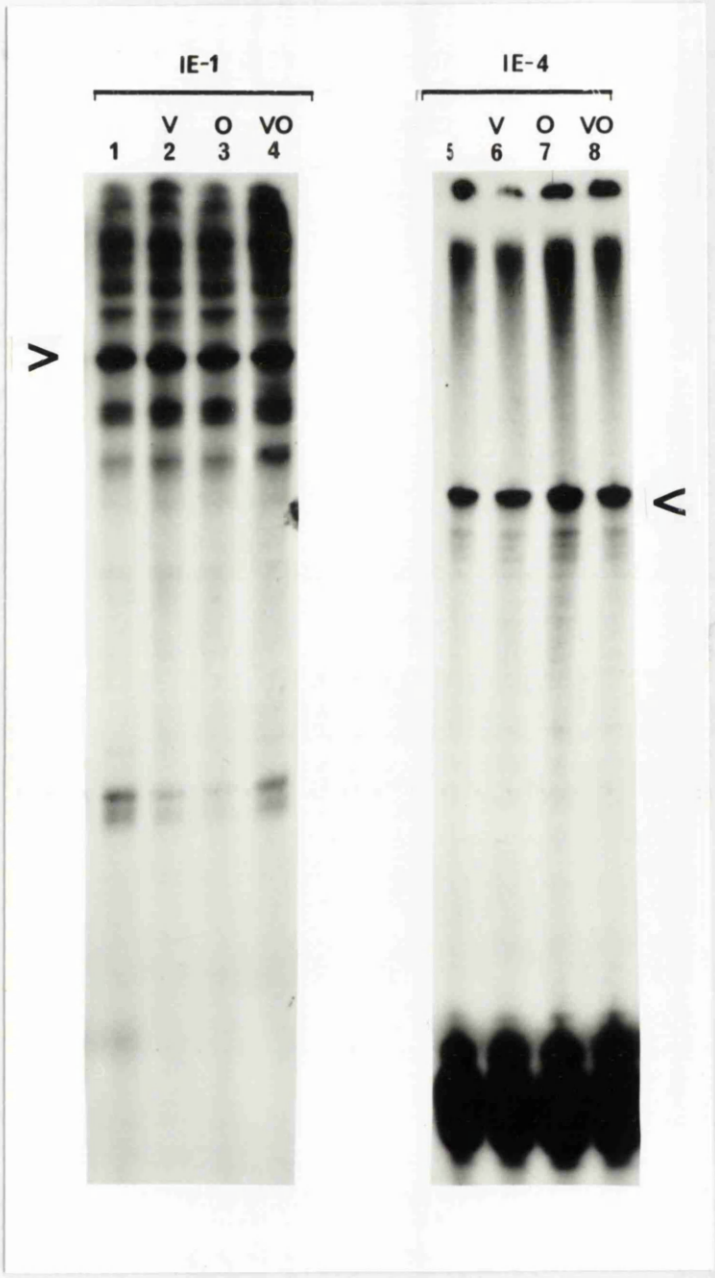
A series of TK$^+$-transformed cell lines were constructed with the HSV-1 TK gene under the control of different classes of viral promoters. The question of specificity of the action of HMBA was addressed using these variants. The parental cell line, the human line 143TK$^-$, was produced by transformation with murine sarcoma virus and made TK$^-$ by selection with 60μg/ml bromodeoxyuridine (Bachetti & Graham, 1977). The mutant *in*1814 was titrated on 143TK$^-$ cells in the presence of HMBA and shown to respond to HMBA in the same manner as HFL cells. The titre of *in*1814 on 143TK$^-$ cells rose from 3.5 x 10^7 pfu/ml in the absence of HMBA to 1.6 x 10^9 pfu/ml in the presence of 5mM HMBA.

The following plasmid constructs were cleaved with *Bam*HI and separately transformed into the parental 143TK$^-$ cell line :- (i) pTK1, which contains the coding and regulatory sequences of the HSV-1 TK gene; (ii) pTKN, which consists of the HSV-1 IE-3 promoter linked to the TK coding sequences; (iii) pTKTaqA, which contains the IE-4 promoter linked to the TK gene. The structures of these plasmid constructs used to generate the

Figure 4.21 Effect of *in vitro* synthesized Vmw65 and Oct-POU domain of Oct-1 on transcription *in vitro*

In vitro transcription reactions were performed using 8 units of a commercial HeLa cell nuclear extract with either 0.1mg pA736 containing the Vmw110 promoter, cleaved with *Nde*I (IE-1; lanes 1-4) or 0.1mg pLWFU17 containing the IE-4 promoter (IE-4; lanes 5-8) in the presence of 15μg Vmw65 (V, lanes 2 and 6) or 15μg Oct-POU domain of Oct-1 (O, lanes 3 and 7) or both (lanes 4 and 8).

Figure 4.21



transformed cell lines are shown in appendix 2. This was achieved by cleaving pTK1, pTKN and pTKTaqA with *Bam*HI, then introducing 1.25μg of the resulting linear plasmid DNA in conjunction with 8.75μg TK^- BHK cell carrier DNA, into 143TK^- cells by calcium phosphate-mediated transfection, in the absence of a DMSO boost (Corsalo & Pearson, 1981). The calcium phosphate/DNA precipitate was added to the overlying medium, and the monolayers were incubated for 24h at 37°C. The non-selective medium was subsequently replaced with HAT selective medium, and changed every 24h during the first 4 days, then every second day. Under HAT selective conditions, only the TK^+ transformed cells survived, generating the mixed cell culture lines 143TK1-1 to 143TK1-4, 143TKN-1 to 143TKN-4 and 143TKTaqA-1 to 143TaqA-4.

4.6.2 Effect of HMBA on TK activity

Using the transformed cell lines 143TK1, 143TKN and 143TKTaqA, it was possible to determine whether the TK, IE-3 or IE-4 promoters, respectively, are responsive to HMBA when resident in the cellular genome. Cell extracts were made from mock-infected untreated cells, and from cells exposed to HMBA for 4h and 8h. Monolayers were infected with 10^7 particles of UV-irradiated *ts*K at 38.5°C and cell extracts were prepared at 4h post-infection. At the NPT, infection with UV-irradiated *ts*K provides Vmw65, which will induce expression from the TAATGARAT motif. Extracts were also made from 143TK1 cell lines infected at 37°C with 20 pfu/cell TK^- HSV-1, at 6h post-infection. Infection with a TK^- virus provides the IE proteins required to transactivate expression from an early promoter. Cell extracts were also prepared from the parental 143TK^- cell line under the conditions specified for the transformed cells. The protein concentration of each cell extract was determined before carrying out TK assays (Table 4.4).

The results from the TK assays confirm that both the IE-3 promoter, (in the 143TKN cell lines), and the IE-4 promoter, (in the 143TaqA cell lines), were responsive to transactivation by Vmw65. This responsiveness to Vmw65 was demonstrated by an increase in TK activity after infection with UV-irradiated *ts*K at the NPT. As expected, expression from the early TK promoter, (in the 143TK1 cell lines), was not induced by infection with UV-irradiated *ts*K at the NPT. Infection of the 143TK1 cell lines with a TK^- virus did however demonstrate that the early TK promoter is responsive to transactivation by IE proteins (Table 4.4). Extracts were also prepared from the parental 143TK^- cell line (Table 4.4). As predicted, 143TK^- extracts did not display any

Table 4.4 Effect of HMBA on TK activity

		TK activity(c.p.m./μg extract)				
Cell line		**M**	**4h**	**8h**	**UV*ts*K**	**TK-**
143TKN	1	17835	26599	30830	86697	
	2	20230	26516	37336	60684	
	3	9811	19628	9877	78884	
	4	16366	19362	23325	66667	
143TK1	1	31172	21385	31527	13486	37575
	2	15802	15859	14874	12113	39492
	3	18485	16254	17150	14784	33560
	4	11718	13418	16065	9456	31713
143TK-	1	5071	26714	13466	50200	
TaqA	2	6637	10154	6607	27019	
	3	15877	26458	14395	34422	
	4	12413	17260	12870	23797	
143TK-		441	468	479	473	
HeLa		7824	7645	7284	ND	

Extracts were prepared from untreated (M) 143TKN, 143TK1, 143TKTaqA, 143TK- and HeLa cells, after exposure to 5mM HMBA for 4h (4h) and 8h (8h) and 8h after infection with 10^7 particles ultraviolet light-inactivated *ts*K at 38.5°C (UV*ts*K). 143TK1 cells were infected 20pfu/cell TK- HSV-1 at 37oC and extracts prepared at 6h post-infection. TK activity was determined by assay of the cytoplasmic fraction from each sample at 30°C for 1h.

The TK+ transformed cell lines are derived from mixed cell cultures.

Table 4.5 Effect of HMBA on TK activity

	TK activity(c.p.m./μg extract)			
Cell line	**MOCK**	**24h HMBA**	**T**	**P**
pTK1-2[a]	33581	61390	3.05 (4)	<0.05
	20096	41216		
	21837	36257		
	34227	36012		
	14949	21643		
pTK1-3[a]	32355	48376	0.015 (4)	N.S.
	64006	44657		
	44487	35242		
	37732	56313		
	38032	32565		
pTKN-2[a]	24323	38031	0.62 (4)	N.S
	34836	79466		
	38510	74163		
	45511	44418		
	83678	39294		
pTKN-2[b]	5118	11262	2.433 (4)	S
	3032	11777		
	3807	5164		
	5994	16616		
	5930	5223		
pTKN-4[a]	6923	39052	2.98 (3)	0.1-0.05
	11262	34005		
	18608	39579		
	26296	26668		
pTKTaqA-3[a]	32355	48376	2.65 (4)	N.S.
	64006	44657		
	44487	35242		
	37732	56313		
	38032	32565		

pTKTaqA-4[a]	12185	23086	0.69 (4)	N.S.
	13251	9600		
	11630	21325		
	13103	26216		
	77776	12794		

Extracts were prepared from untreated (MOCK) cells and cells exposed to 5mM HMBA for 24h (24h HMBA). TK activity was determined by assay of the cytoplasmic fraction from each sample at 30°C for either 1h ([a]) or 15 min ([b]). A T-test (T) was employed to determine whether there is a significant difference between the untreated cells and those exposed to HMBA. The number of degrees of freedom are indicated in brackets. With four degrees of freedom, any value greater than 2.78 was regarded as significant. The probability (P) of these TK activity values not being significantly different is noted (N.S.= not significant; S= significant).

The TK+ transformed cell lines are derived from mixed cell cultures.

significant TK activity either alone, after exposure to HMBA or when infected with UV-irradiated *ts*K (Table 4.4).

Analysis of the results (Table 4.4) suggests that the 143TKN and 143TaqA cell lines showed a slight increase in TK activity after exposure to HMBA, while the 143TK1 cell lines did not appear to respond at all. However, the TK activities obtained after HMBA treatment did not approach the significantly higher levels obtained after induction by UV-irradiated *ts*K. TK assays were also performed using HeLa cell extracts treated with HMBA for 0, 4 and 8h, in the absence of 50μM dTTP in the reaction mix, to determine whether the resident TK promoter in the cellular genome is responsive to activation by HMBA (Table 4.4). Unlike HSV encoded TK, cellular TK is inhibited by 50μM dTTP (Jamieson & Subak-Sharpe, 1974). The TK activities obtained demonstrate that the HeLa cell TK promoter is not activated by HMBA.

Given that an 8h exposure to HMBA may not be sufficient to increase TK activity significantly, the time of exposure to HMBA was increased to 24h. In addition, to ensure that any variations in TK activity are real and not a consequence of random variations between samples, five mock and five HMBA-treated extracts were prepared from each cell line (Table 4.5). Within each set of results, obtained under identical conditions, the TK activity varied approximately twofold. Despite this variation, it is clear that exposure to HMBA for 24h did not dramatically increase the level of expression from the IE-3, IE-4 and TK promoters in the cellular genome. This conclusion is despite observations that these promoters are responsive to HMBA in plasmid-based CAT assays (Figure 4.7) and that the IE-3 and IE-4 RNA levels during *in*1814 infection are increased in the presence of HMBA (Figure 4.5). The variations in TK activities between different isolates may represent differences in the number of copies of the TK gene integrated into the cellular genome and/or the effect of the position of the integrated gene on its level of expression. Despite the small sample size and variation in TK activity, it is possible to employ a "T-test" to determine whether the differences between the sets of HMBA-treated and untreated extracts are statistically different from each other. With four degrees of freedom, any value of T greater than 2.78 was regarded as significant. Although, no significant difference in TK activities was seen between mock and HMBA-treated extracts prepared from 143TK1, 143TKN and 143TKTaqA cells (Table 4.5), it is certainly possible that a larger sample size would reveal small but significant differences between the samples. Northern blot analysis of RNA extracted from each of the transformed cell lines, after 4h exposure to HMBA and mock treatment, did

not reveal any obvious increase in RNA produced in the presence of 5mM HMBA (Results not shown).

Once integrated into the cellular genome, the IE-3, IE-4 and TK promoters lose their ability to respond to induction by HMBA, although the IE-3 and IE-4 promoters are still responsive to transinduction by Vmw65 and the TK promoter can be activated by IE proteins. Therefore, although when in the cellular genome these promoters are no longer responsive to HMBA, they are still activated by the appropriate viral transactivators. Clearly exposure to HMBA does not result in a gross increase in expression of all cellular genes but has an effect on specific, but as yet unidentified, genes. The results obtained suggest that the effect of HMBA on a particular promoter sequence, varies depending on the structural environment of the promoter.

4.7 Effects of the metabolites of HMBA on *in*1814

A number of known metabolites of HMBA have been tested for their ability to induce differentiation of both MELCs and HL60 cells independently and in combination with HMBA (Reuben *et al.*, 1976, 1978, 1980; Meilhoc *et al.*, 1986; Snyder *et al.*, 1988). The primary metabolite, N-acetyl-1,6-diaminohexane, is a more potent inducer of HL60 cell differentiation than the parental molecule HMBA. The metabolites AmHA and AcHA both failed to induce differentiation of either MELCs or HL60 cells, but did potentiate the induction of differentiation of HL60 cells by HMBA, when used in conjunction with HMBA. In contrast, DAH did not induce differentiation either alone or in combination with HMBA (Snyder *et al.*, 1988). Experiments were carried out to determine whether any of the metabolites of HMBA increase the titre of *in*1814.

Unfortunately, as a consequence of its extreme toxicity, N-acetyl-1,6-diaminohexane was not available for analysis. Titrations of *in*1814 were carried out on HFL cells in the presence of various concentrations of AmHA and AcHA, individually (Table 4.6a) and in combination with 1 (Table 4.6b) and 3mM (Table 4.6c) HMBA. At 2 days post-infection, cells were fixed and stained, and the resulting titres determined. From these results, it was concluded that exposure to metabolites AmHA, AcHA and DAH does not have the same dramatic effect on *in*1814 titre as does exposure to HMBA. In addition, titration of *in*1814 in the presence of HMBA and in conjunction with AmHA, AcHA or DAH does not enhance the HMBA-mediated effect on the titre of *in*1814. As HMBA treatment is required immediately after infection for 3 to 4h for maximum effect, it would depend on how rapidly HMBA is metabolized

Table 4.6 Effects of metabolites of HMBA on the titre of *in*1814

(a)

Concentration (mM)	Titre (pfu/ml) HMBA	AmHA	AcHA	DAH
0	5.0 X 10^6	1.0 X 10^7	1.0 X 10^7	5.0 X 10^6
0.5	1.0 X 10^8	5.0 X 10^6	5.0 X 10^6	TOXIC
1	2.0 X 10^8	1.5 X 10^7	5.0 X 10^6	TOXIC
3	2.7 X 10^8	5.0 X 10^6	5.0 X 10^6	TOXIC
5	3.7 X 10^8	1.5 X 10^7	1.0 X 10^7	TOXIC

(b)

Concentration (mM)	Titre (pfu/ml) + 1mM HMBA AmHA	AcHA	DAH
1	1.3 X 10^8	1.5 X 10^8	4.5 X 10^7
3	1.1 X 10^8	1.3 X 10^8	TOXIC
5	8.0 X 10^7	1.4 X 10^8	TOXIC

(c)

Concentration (mM)	Titre (pfu/ml) + 3mM HMBA AmHA	AcHA	DAH
1	1.1 X 10^8	3.8 X 10^8	7.0 X 10^7
3	2.4 X 10^8	2.2 X 10^8	TOXIC
5	2.9 X 10^8	2.5 X 10^8	TOXIC

Compounds were added after the 1h adsorption and penetration period. Monolayers were fixed and stained 2 days post-infection.

in HFL cells as to whether it is feasible to consider if any of these metabolites actually exert an effect on the titre of *in*1814. The conversion of HMBA to its metabolites may explain why HMBA pre-treatment of cells, prior to *in*1814 infection, has no effect on the subsequent titre.

4.8 Modulation of PKC activity

As the phorbol ester TPA is known to antagonise HMBA-mediated induction of MELC differentiation (Falleto *et al.*, 1985), experiments were carried out to determine whether TPA also inhibits the HMBA-mediated increase in the titre of *in*1814. The mutant *in*1814 and revertant 1814R were titrated in the presence and absence of 5mM HMBA, and in the presence of various concentrations of TPA. Cells were exposed to compounds immediately after the 1h adsorption and penetration period until 48h, when the cells were fixed and stained and the resulting titres determined (Table 4.7). The titres obtained reveal that exposure to TPA did not inhibit the HMBA-mediated increase in the titre of *in*1814. Initial exposure of cells to TPA results in a rise of PKM activity. However, with continued exposure to TPA there is an overall decrease in PKM activity. Although initial exposure of cells to HMBA also results in a rise in PKM activity, there is no dramatic decrease upon prolonged exposure (Section 1.4.4.2). Therefore, to investigate whether the PKM fragment of PKC is responsible for the HMBA-mediated increase in the titre of *in*1814, HFL cells were pretreated with TPA for 24h and the agent was withdrawn immediately prior to infection with *in*1814 or 1814R, in the presence or absence of HMBA (Table 4.8). Pretreatment of cells with TPA for 24h prior to infection with *in*1814, which virtually eliminates PKM activity, did not inhibit the HMBA-mediated increase in *in*1814 titre.

Leupeptin is an inhibitor of the enzyme calpain, responsible for catalysing the conversion of membrane bound PKC to the soluble activated PKM form (Melloni *et al.*, 1982; Pontremoli *et al.*, 1987), which also blocks HMBA-induced MELC differentiation (Melloni *et al.*, 1987). To investigate whether leupeptin specifically inhibits the HMBA-mediated increase in *in*1814 titre, HFL cells were infected with *in*1814 or 1814R in the presence or absence of HMBA. Various concentrations of leupeptin were also added to the overlying medium (Table 4.9). The titres obtained reveal that inhibition of PKM activity, by treatment with leupeptin, does not antagonize the HMBA-mediated effect on *in*1814 titre. Therefore the modulation of PKC does not appear to be responsible for the HMBA-mediated increase in *in*1814 titre.

Table 4.7 Effect of TPA on the titre of *in*1814 and 1814R

Virus	Treatment		Titre (pfu/ml)
*in*1814	-	-	1.0×10^6
*in*1814	-	10^{-9} M TPA	1.8×10^6
*in*1814	5mM HMBA	-	2.3×10^8
*in*1814	5mM HMBA	10^{-9} M TPA	2.6×10^8
*in*1814	5mM HMBA	10^{-9} M TPA	2.4×10^8
*in*1814	5mM HMBA	3×10^{-9} M TPA	2.3×10^8
*in*1814	5mM HMBA	3×10^{-9} M TPA	1.7×10^8
*in*1814	5mM HMBA	6×10^{-9} M TPA	TOXIC
1814R	-	-	8.9×10^8
1814R	-	10^{-9} M TPA	8.7×10^8
1814R	5mM HMBA	-	9.1×10^8
1814R	5mM HMBA	10^{-9} M TPA	8.5×10^8
1814R	5mM HMBA	10^{-9} M TPA	8.8×10^8
1814R	5mM HMBA	3×10^{-9} M TPA	8.6×10^8
1814R	5mM HMBA	3×10^{-9} M TPA	9.4×10^8

Compounds added after the 1h adsorption and penetration period. Monolayers were fixed and stained 2 days post-infection.

Table 4.8 Effect of pre-exposure to TPA on the titre of *in*1814 and 1814R

Virus	Treatment		Titre (pfu/ml)
*in*1814	-	-	1.0×10^6
*in*1814	-	10^{-9} M TPA[a]	$<5.0 \times 10^5$
*in*1814	-	10^{-9} M TPA	2.0×10^6
*in*1814	-	3×10^{-9} M TPA	1.0×10^6
*in*1814	-	3×10^{-9} M TPA	1.5×10^6
*in*1814	5mM HMBA[b]	-	3.1×10^8
*in*1814	5mM HMBA	10^{-9} M TPA	3.8×10^8
*in*1814	5mM HMBA	10^{-9} M TPA	3.4×10^8
*in*1814	5mM HMBA	3×10^{-9} M TPA	3.5×10^8
*in*1814	5mM HMBA	3×10^{-9} M TPA	3.7×10^8
1814R	-	-	9.9×10^8
1814R	-	10^{-9} M TPA	1.1×10^9
1814R	-	10^{-9} M TPA	9.6×10^8
1814R	-	3×10^{-9} M TPA	1.1×10^9
1814R	-	3×10^{-9} M TPA	1.2×10^9
1814R	5mM HMBA	-	1.0×10^9
1814R	5mM HMBA	10^{-9} M TPA	1.1×10^9
1814R	5mM HMBA	10^{-9} M TPA	1.0×10^9
1814R	5mM HMBA	3×10^{-9} M TPA	1.0×10^9
1814R	5mM HMBA	3×10^{-9} M TPA	1.1×10^9

[a] TPA present for 24h immediately prior to infection. The medium containing the TPA was removed, monolayers were washed twice with EHu5 and overlaid with EHu5.

[b] HMBA present after adsorption and penetration

Monolayers were fixed and stained 2 days post-infection.

Table 4.9 Effect of leupeptin on the titre of *in*1814 and 18164R

Virus	Treatment		Titre (pfu/ml)
*in*1814	-	-	$<5.0 \times 10^6$
*in*1814	-	10^{-9} M leupeptin	$<5.0 \times 10^6$
*in*1814	5mM HMBA	-	2.7×10^8
*in*1814	5mM HMBA	10^{-8} M leupeptin	5.6×10^8
*in*1814	5mM HMBA	10^{-8} M leupeptin	3.4×10^8
*in*1814	5mM HMBA	10^{-9} M leupeptin	1.7×10^8
*in*1814	5mM HMBA	10^{-9} M leupeptin	1.7×10^8
1814R	-	-	8.1×10^8
1814R	-	10^{-9} M leupeptin	8.1×10^8
1814R	5mM HMBA	-	1.4×10^9
1814R	5mM HMBA	10^{-8} M leupeptin	1.1×10^9
1814R	5mM HMBA	10^{-8} M leupeptin	1.2×10^9
1814R	5mM HMBA	10^{-9} M leupeptin	1.2×10^9
1814R	5mM HMBA	10^{-9} M leupeptin	1.0×10^9

Compounds added after the 1h adsorption and penetration period. Monolayers were fixed and stained 2 days post-infection.

HMBA-mediated MELC differentiation is a complex multistep process (Chen *et al.*, 1982) and although TPA inhibits commitment to HMBA-induced differentiation, it does not prevent the early HMBA-induced changes in *c-myc*, *c-myb* and *c-fos* expression. These early changes also occur in the presence of cycloheximide and may therefore reflect alterations in the state of phosphorylation of proteins involved in the regulation of these genes. Exposure of HFL cells to TPA, both prior to and post infection, did not significantly antagonize the HMBA-mediated effect on the titre of *in*1814. It is therefore feasible that these early alterations in gene expression in response to HMBA, prior to the involvement of PKC, mediate the effect on *in*1814 titre. Consequently, the HMBA-mediated increase in *in*1814 titre may provide a model system for investigating the very early or initial events involved in HMBA-induced differentiation of MELCs.

4.9 The effect of HMBA exposure at different stages of calcium phosphate-mediated transfection

Various modifications to the calcium phosphate-mediated transfection procedure (Stow & Wilkie, 1986) were introduced, to determine whether HMBA could functionally replace or have an additive effect in conjunction with the DMSO boost. 0.5µg HSV-1 DNA was introduced into BHK cells using the modified protocols detailed in Table 4.10a. Since 1.5% (v/v) [200mM] DMSO is equivalent to 5mM HMBA during MELC differentiation then, 25% DMSO is equivalent to 83mM HMBA during calcium phosphate-mediated transfection. However, the equivalent concentration of HMBA (83mM) was unable to functionally substitute for the 25% DMSO boost in the transfection procedure. However, addition of 5mM HMBA to the overlying medium after the DMSO boost apparently increased the efficiency of transfection, at least with respect to the number of plaques obtained.

The effect of exposure to HMBA on the plaquing of both wild type HSV-1 and *in*1814 DNA was subsequently investigated. Different concentrations of either wild type HSV-1 or *in*1814 DNA were introduced into BHK cells by calcium phosphate-mediated transfection. After the DMSO boost, 5mM HMBA was added to the overlying medium, and the cells were fixed and stained 2 days later (Table 4.10b). The addition of 5mM HMBA to the culture medium after the DMSO boost, increases the number of plaques obtained for both *in*1814 and wild type HSV-1 DNA and can therefore also compensate for the absence of the potent transactivator Vmw65 during transfection procedures. The observation that HMBA can also increase the number of

Table 4.10a Effect of exposure to HMBA during calcium phosphate-mediated transfection of viral DNA into BHK cells

Modified protocol	No. plaques
Normal protocol	119
Minus DMSO boost	0
"HMBA" boost replacing DMSO boost	0
HMBA during calcium phosphate precipitation, up to & after boost	312
HMBA added after DMSO boost	322

Monolayers were fixed and stained two days post-transfection.

Table 4.10b Effect of addition of HMBA after DMSO boost on plaque formation

	No. plaques				
	WT		*in*1814		
µg DNA	-	+	-	+	HMBA
0.3	40	133	56	137	
0.1	26	66	14	69	
0.03	1	17	1	35	
0.01	0	4	0	1	
0.003	0	0	0	0	

5mM HMBA was added to the culture medium after the 4min 25% DMSO boost. Monolayers were fixed and stained 2 days post-transfection.

plaques obtained after transfection of viral DNA implies that HMBA is not involved in mediating the viral uncoating process during infection.

4.10 Effect of HMBA on Ad E1a deletion mutant, *dl*312

Adenovirus E1a gene product is a potent and promiscuous transactivator of gene expression (Berk, 1986; Flink & Shenk, 1990) required for the positive regulation of early adenovirus gene expression (Jones & Shenk, 1979) and is composed of three regions, namely 1, 2 and 3 (Moran & Mathews, 1987). Using GAL4-E1a fusion proteins, Lillie and Green (1989) demonstrated that region 3 contains both a transcriptional activation domain and a promoter binding activity. E1a, like Vmw65, is not a sequence-specific DNA binding protein (Ferguson *et al.*, 1985) and the E1a activating region can substitute for the acidic activating region of the yeast activator GAL4 (Ptashne, 1988; Mitchell & Tjian, 1989; Lillie & Green, 1989). As a consequence of these apparent similarities between Vmw65 and E1a, titration experiments were performed to determine whether HMBA can also complement the replication of an Ad5 E1a deletion mutant *dl*312 (Jones & Shenk, 1979).

Wild type Ad5 and *dl*312 were titrated on HeLa cells in the presence of 5mM HMBA for 24h post infection. At day 10, cells were fixed and stained and the titres determined (Table 4.11a). Exposure to 5mM HMBA for 24h had no effect on the titre of wild type Ad5, but resulted in a small rise in the titre of *dl*312. However, as *dl*312 forms plaques inefficiently on HeLa cells due to the lack of functional E1a, it is possible that not all the progeny viruses were detected. In addition, the time of exposure to HMBA may not be the optimum required as the replication cycle of adenoviruses is considerably longer than that of HSV-1. Therefore, *dl*312 was titrated on HeLa cells, in the presence or absence of 5mM HMBA for 48h. The progeny viruses were then harvested and titrated on 293 cells, which constitutively express E1a (Graham *et al.*, 1977), thus allowing all potential *dl*312 plaques to be detected (Table 4.11b). Despite an approximate 10-fold increase in the titre of *dl*312, HMBA treatment did not fully compensate for the lack of E1a activity and did not act as, nor induce, a cellular homologue of E1a. However, these results clearly suggest that HMBA affects gene expression of a non-herpesvirus, thus indicating a more universal mode of action. In addition, titration of *in*1814 on 293 cells, which express E1a, did not enhance the resulting titre (results not shown).

Table 4.11a Effect of HMBA on the titre of Ad5 and *dl*312 on HeLa cells

	No. plaques				
	Ad5 WT			*dl*312	
pfu/cell	-	+	pfu/cell	-	+ HMBA
0.0001	42	49	0.01	1	5
0.0002	47	47	0.02	2	8
0.0004	cpe	cpe	0.05	4	19
0.001	cpe	cpe	0.075	4	34

5mM HMBA added to culture medium after the 1h adsorption and penetration period and retained for 24h then replaced by an agar overlay. Monolayers were fixed and stained 10 days post-infection and the resulting titre determined.

Table 4.11b Effect of HMBA on the titre of *dl*312 on 293 cells

Titre *dl*312 (pfu/ml)	
-HMBA	+HMBA
1.3×10^3	3.5×10^4
2.5×10^3	3.7×10^4
5.0×10^3	7.2×10^4
1.4×10^4	1.3×10^5

5mM HMBA added to culture medium after the 1h adsorption and penetration period and retained for 48h. Progeny viruses were harvested and titrated on 293 cells. Monolayers were fixed and stained 10 days post-infection and the resulting titre determined.

5. DISCUSSION

The experiments described in this thesis demonstrate that a dramatic reversal of the phenotype of the Vmw65 insertion mutant *in*1814 (Ace *et al.*, 1989) can be achieved by the addition of 5mM HMBA to the cell culture medium. At this concentration, although cell division appears to cease, the agent is not cytotoxic and may be used on a variety of cell types. Apart from the theoretical considerations, the effect of HMBA is of practical value, since a short exposure of the agent allows *in*1814 to be propagated and titrated almost as efficiently as wild type HSV-1. Previously, complementation with ultraviolet light-inactivated HSV, a more cumbersome process, was necessary to assay *in*1814 particle production by titration (Ace *et al.*, 1989; Steiner *et al.*, 1990).

5.1 Relationship between the "HMBA effect" on *in*1814 titre and HMBA-induced MELC differentiation

The fact that HMBA, DMSO and hypoxanthine are all inducers of MELC differentiation (Friend *et al.*, 1971; Reuben *et al.*, 1976), and that the optimum concentrations for complementation of *in*1814 and differentiation (Reuben *et al.*, 1980) are equivalent, suggests that a common pathway underlies the two phenomena. In addition, both DMSO and hypoxanthine significantly increase the titre of *in*1814, however, they are not as effective as HMBA. This is consistent with the fact that HMBA is a more potent inducer of MELC differentiation than DMSO, acting in a concentration range 50 times lower (Palfrey *et al.*, 1977). In addition, the effect on *in*1814 requires only a short exposure to HMBA (1 to 5h, Figure 4.3) whereas a longer treatment, greater than 12h, in combination with a period of protein synthesis is necessary for irreversible commitment to differentiation (Marks *et al.*, 1987).

The effect of HMBA on the titre of *in*1814 is not specific to HFL cells, being reproducible in BHK and HeLa cells (Figure 4.1). This finding suggests that HMBA affects or functions through a ubiquitous phenomenon or protein. In addition, the HMBA effect is presumably on pre-existing cellular or viral components, since the agent is ineffective if added and withdrawn immediately prior to infection, but can act on IE RNA levels in the presence of cycloheximide (Figure 4.5). Modification of proteins, perhaps as a consequence of alterations in PKC levels (Melloni *et al.*, 1987), is a plausible

* On the assumption that the alterations in proto-oncogene expression in MELCs are the same as the changes in HFL cells in response to HMBA.

mode of action, but it is equally possible that the compound, or metabolites derived from it, act directly on cellular or viral macromolecules.

The phorbol ester TPA, a known antagonist of HMBA-mediated MELC differentiation which induces a transient rise in PKM activity (Falleto *et al.*, 1985), fails to inhibit the HMBA-mediated increase in *in*1814 titre when added in conjunction with HMBA after the 1h adsorption and penetration period. Depletion of PKM activity, by prolonged exposure to TPA, also fails to prevent the HMBA-mediated increase in *in*1814 titre. In addition, exposure to leupeptin, which inhibits the conversion of membrane bound inactive PKC to the soluble activated PKM form (Melloni *et al.*, 1982; Pontremoli *et al.*, 1987) also fails to antagonize the effect of HMBA on *in*1814. Therefore, HMBA-mediated modulation of PKC activity does not appear to be responsible for the effect on *in*1814 titre.

To reconcile the fact that TPA, a compound known to antagonize inducer-mediated differentiation, does not block the HMBA-mediated effect on *in*1814 titre, the following points must be taken into consideration. Firstly, despite TPA inhibiting the commitment of MELCs to differentiation, TPA does not prevent the early HMBA-induced changes in *c-myc*, *c-myb* and *c-fos* expression (Melloni *et al.*, 1987). It is therefore feasible that alterations in the expression of these oncogenes may be involved in the HMBA-mediated increase in *in*1814 titre. Secondly, it is known that HMBA-induced early changes in protooncogene expression also occur in the presence of cycloheximide and may therefore reflect alterations in the state of phosphorylation of proteins involved in the regulation of these genes. These same putative regulatory factor(s) may be responsible for the increased accumulation of *in*1814 IE RNA. Indeed, a number of studies imply that transcription factors may exist in an inactive state prior to post-translational modification (Imbra & Kann, 1986), thus providing a potential mechanism whereby HMBA could function. To investigate further whether any cellular or viral proteins are specifically phosphorylated in response to HMBA, proteins could be labelled with [^{32}P]-orthophosphate in the presence and absence of HMBA.

The decrease in *c-myc* mRNA occurs within 1h exposure to HMBA, while the alterations in *c-myb* and *c-fos* levels occur by 4h (Ramsay *et al.*, 1986; Richon *et al.*, 1989). ✳, it is more likely that the decrease in *c-myc* levels, or the activity of a factor(s) controlling this effect, is involved in the HMBA-mediated increase in *in*1814 titre, considering only 1h exposure results in a large increase in titre. To investigate this possibility, future work could involve the introduction of a plasmid expressing anti-sense *c-myc* RNA

into HFL cells prior to infection with *in*1814 or 1814R, in the absence of HMBA. If the increase in *in*1814 titre in the presence of HMBA occurs as a direct consequence of the reduced *c-myc* RNA levels, then one would expect to detect a rise in titre similar to that observed with HMBA treatment. If however, the sequestering of *c-myc* mRNA had no specific effect on the titre of *in*1814, it would be more likely that a factor(s) regulating *c-myc* expression is also involved in the modulation of *in*1814 titre. Darling *et al.* (1989) demonstrated that DMSO treatment did not induce a rapid transient reduction in the steady state levels of *c-myc* in HeLa cells. Therefore, as the HMBA effect on *in*1814 titre is observed in HeLa cells, it is unlikely that the reduction in *c-myc* mRNA is directly responsible.

Like TPA, another inhibitor of HMBA-induced differentiation, dexamethasone, also results in a block at the late stage and has no effect on the early pre-commitment events (Santoro *et al.*, 1978). It may prove illuminating to determine whether, like TPA, dexamethasone also fails to inhibit the HMBA-mediated increase in the titre of *in*1814.

As the effect of HMBA on *in*1814 is immediate and given that PKC inhibitors, which function further along the multistep pathway, do not antagonize the HMBA-mediated increase in *in*1814 titre, it seems likely that the early precommitment events of HMBA-induced differentiation are being detected in this system.

It has been proposed that vincristine-resistant MELC variants, which display accelerated HMBA-mediated differentiation, display constitutive expression of a factor(s) critical, and rate-limiting, for early events in commitment (Melloni *et al.*, 1988; Richon *et al.*, 1991). If MELCs prove to be permissive for HSV infection, assays may be carried out to determine whether *in*1814 has a greater titre on the vincristine-resistant cell lines than on the normal MELCs. Identification and isolation of this putative factor(s) would facilitate the titration of *in*1814 in its presence to determine whether this activity alone was sufficient to emulate the effect observed with HMBA treatment. In addition, nuclear extracts could be prepared from normal uninduced MELCs and from vincristine-resistant variants, to allow the comparison of complex formation, by gel retardation analysis, on a number of promoter motifs, including the TAATGARAT and TATA box.

As HMBA, is a potent hypomethylating agent, it may affect gene expression by altering the degree of methylation of particular genes. Demethylation of cytosine residues within the dinucleotide sequence CpG has been associated with transcriptional inactivity (Bird, 1986). Treatment of cells with the demethylating agent, 5-azacytosine, often leads to the

reactivation of genes that were both methylated and transcriptionally repressed (Mohandas *et al.*, 1981; Venolia *et al.*, 1982). However, demethylation of DNA by HMBA is unlikely to be involved in the activity on *in*1814, for a number of reasons. Firstly, HSV DNA is not methylated (Low *et al.*, 1969), thus a direct effect on the template is improbable. Secondly, it is difficult to envisage how demethylation of cell DNA without subsequent protein synthesis could affect HSV gene expression. Thirdly, loss of 5-methylcytosine in MELCs does not commence until 3h after the addition of HMBA and reaches a maximum level at 6h (Razin et al., 1988), yet 3h exposure is sufficient to give an almost complete increase in *in*1814 titre. Finally, 5-azacytidine did not mimic the effect of HMBA but reduced the titre of *in*1814.

The primary metabolite, N-acetyl-1,6-diaminohexane, is a more potent inducer of HL60 differentiation than the parent compound HMBA (Snyder *et al.*, 1988), but is unfortunately not commercially available. If this compound was available, then *in*1814 may be titrated in the presence of various concentrations of N-acetyl-1,6-diaminohexane to determine whether it has any effect on the titre. None of the other metabolites of HMBA tested, namely AmHA, AcHA and DAH, facilitated a dramatic increase in the titre of *in*1814. In view of the observation that the HMBA-mediated increase in *in*1814 titre is an immediate response, it is likely that the action of HMBA itself, or possibly N-acetyl-1,6-diaminohexane, depending on the rate of metabolism in HFL cells, is responsible for the dramatic increase in *in*1814 titre. Pre-exposure of HFL cells to HMBA prior to *in*1814 infection had no effect on the subsequent titre. This may be explained by the rapid conversion of HMBA to an inactive metabolite(s).

If the effect of HMBA on the titre of *in*1814 occurs by the same mechanism as induced MELC differentiation, then it may be used as a simple system for rapidly screening related compounds for their ability to induce differentiation. A series of extremely potent differentiation inducing agents, which are structurally related to HMBA, have recently been synthesized (Breslow *et al.*, 1991). Differentiation-inducing assays have revealed that related inducers with flexible linear structures are active differentiators of MELCs, HL-60 and HT-29 cells, being one hundred times more potent than HMBA. In contrast, rigid compounds, containing benzene ring spacers, show greater activity towards MELCs, while being much less active towards HL-60 cells. Furthermore, as the rigid compounds have different geometric requirements and are not additive with HMBA in their effect, Breslow *et al.* (1991) proposed that rigid compounds have a different site of action from flexible inducers. If these compounds were made available, titrations of

*in*1814 in the presence of agents belonging to each mechanistic group should be undertaken. If functional distinctions could also be made between the rigid and flexible inducers with respect to their effect in the titre of *in*1814, then this assay may form the basis of a rapid screen facilitating the identification of novel inducers of differentiation. It is likely that variations in the potency of compounds could be detected as hypoxanthine, a less potent inducer of differentiation, is also less effective than HMBA at complementing *in*1814. Characterization of the effect of compounds falling into two different mechanistic groups on *in*1814 titre, may provide greater insight into the mechanism of action of HMBA on the titre of *in*1814, reactivation of latent HSV and differentiation.

As the uptake of virus DNA to the nucleus was not altered by HMBA, the most likely events affected are uncoating or IE transcription. Little is known in detail about the mechanism of uncoating, but an hypothesis that HMBA affects this process presupposes that *in*1814 is defective in uncoating. Previous studies have shown that *in*1814 can be complemented completely by the supply of Vmw65 in *trans* and partially by the provision of Vmw110 through plasmid transfections (Ace *et al.*, 1989), observations that are difficult to reconcile with an uncoating defect. In addition, the demonstration that HMBA treatment increases the efficiency of plaque formation after the introduction of both *in*1814 and wild type HSV-1 DNA by calcium phosphate-mediated transfection (Section 4.9) argues against HMBA being involved in the viral uncoating process. Futhermore, in view of the involvement of Vmw65 in the activation of IE transcription, it is most likely that the reduced accumulation of IE RNA observed after infection with *in*1814 is due to impairment of transcription.

5.2 Does HMBA activate a cellular homologue of a viral transactivator?

It is feasible that the viral transactivator Vmw175 could be a target for post-transcriptional modifications by the action of HMBA as it is known to be phosphorylated at serine and threonine residues and poly(ADP-ribosyl)ated (Powell & Purifoy, 1976; Pereira *et al.*, 1977; Cabral *et al.*, 1980; Hay & Hay, 1980; Preston & Notarianni, 1983; Faber & Wilcox, 1986a; Blaho *et al.*, 1992). Furthermore, Michael *et al.* (1988) demonstrated that different electrophoretic forms of Vmw175 differ in their ability to bind specific DNA sequences. Therefore, if a cellular factor functionally similar to Vmw175 existed, then it would be likely that its activity would be controlled in a

similar manner. However, HMBA does not complement the growth of the mutant *in*1411, which lacks a functional Vmw175, in BHK or HFL cells, and therefore does not act as or induce an active cellular homologue of this essential viral transactivator. Further evidence supporting this hypothesis may be obtained if TK assays were performed and total protein synthesis examined, using untreated and HMBA-treated *in*1411-infected cell extracts.

An alternative hypothesis is the possibility that HMBA functions through a cellular homologue of the potent promiscuous transactivator Vmw110 (Everett, 1984; O'Hare & Hayward, 1985; Gelman & Silverstein, 1985, Quinlan & Knipe, 1985) which could be responsible for the activation of IE gene expression in the absence of Vmw65. There are a number of lines of evidence which suggest the existence of such a protein. *In vitro*, latent *dl*1403, lacking a functional Vmw110 (Stow & Stow, 1986), can be reactivated by superinfection with HCMV in a situation where no Vmw110 is present (Russell *et al.*, 1987). When Harris *et al.* (1989) later demonstrated that Vmw110 alone was sufficient to reactivate latent HSV-2 from latency *in vitro*, this observation raised the possibility of the existence of a cellular homologue substituting for the function of Vmw110. Futhermore, a variant cell line, 2.5BHK, has been isolated which supports the replication of *dl*1403 to a greater extent than the parental BHK cell line (Everett, 1989). However, this is unlikely to be the mechanism whereby HMBA increases the titre of *in*1814 for a number of reasons. Firstly, the provision of Vmw110 *in trans* during *in*1814 infection does not fully compensate for the lack of Vmw65, resulting in only an approximate 10-fold increase in titre (Ace *et al.*, 1989), compared with the 250-fold increase in the presence of HMBA. However, the effectivness of this experiment is limited by the efficiency of transfection. In addition, titration of Vmw110 deficient mutants *dl*1403 and *in*1825 in the presence of HMBA results in limited sevenfold and fourfold increases respectively (Section 4.2.2), demonstrating that HMBA treatment does not substantially compensate for the absence of Vmw110.

The simplest explanation for the effect of HMBA on the titre of *in*1814 is that HMBA compensates for the lack of functional Vmw65, either directly or via the modulation of one or more cellular factors. As for Vmw110, there is also evidence supporting the existence of a putative cellular protein which is functionally equivalent to Vmw65. It seems unlikely that an alternative virion component exists which is functionally similar to Vmw65, in view of the fact that PRV fails to stimulate HSV IE gene expression in the presence of cycloheximide (Campbell *et al.*, 1987) and because of the absence of a

functional homologue to Vmw65 in the VZV genome (Davison & Scott, 1987; McKee *et al.*, 1990).

Stimulation of transcription through the octamer motif, in the absence of Vmw65, occurs in the cell cycle dependent regulation of the H2b gene (La Bella *et al.*, 1988). HSV IE gene expression has also been shown to vary during the cell cycle, as the mutant *in*1814 forms plaques less efficiently on HeLa cells synchronised at the G_2 phase of the cell cycle than at G_1/S, demonstrating an increased requirement for Vmw65 when cultures reached the G_2 phase (Daksis and Preston, 1992). Interestingly, treatment of cells with DMSO and HMBA is known to prolong the G_1 phase (Terada *et al.*, 1977; Friedman & Schildkraut, 1978), during which *in*1814 has been shown to plaque most efficiently. It is possible that the lengthened G_1 phase may lead to either the accumulation or increased activity of a cellular factor involved in the HMBA-mediated effect on *in*1814 titre. As the Oct-1 motif is required for the efficient *in vitro* transcription using S-phase, but not G_1 phase extracts (Fletcher *et al.*, 1987) it is feasible that this effect represents the differential expression of Oct-1 or other cellular transcription factors, during the cell cycle. Extracts prepared from cells synchronised at different stages of the cell cycle could therefore be used to determine whether the degree of the HMBA-mediated rise in *in*1814 titre varies during the cell cycle.

Despite the potential existence of a Vmw65 functional equivalent homologue, the inability of HMBA treatment to induce IEC formation in the absence of functional Vmw65, leads to the conclusion that the compound neither acts as, nor activates, a cellular homologue of Vmw65 (Section 4.3.1). In addition, the observation that the inclusion of 5mM HMBA in the binding assays neither promotes IEC formation with *in*1814-infected nuclear extract nor *in*1814 virion extract, reveals that the HMBA molecule does not function as a linker bringing together Oct-1 and the mutant Vmw65. There must be an alternative mechanism whereby HMBA promotes IE gene expression during *in*1814 infection.

The adenovirus transactivator, E1a, shares a number of functional similaraties with HSV-1 Vmw65. For example the activating regions of both Vmw65 and E1a can be substituted for the acidic activation domain of GAL4 (Ptashne, 1988; Mitchell & Tjian, 1989; Lillie & Green, 1989). In addition E1a, like Vmw65, has an indirect mechanism of transactivation which is mediated through protein protein interactions with cellular components (Ferguson *et al.*, 1985; Nevins, 1989). Titration of an Ad E1a deletion mutant, *dl*312, in the presence of HMBA revealed that HMBA treatment does not fully compensate for the lack of E1a activity. Therefore, HMBA does not result in the activation

of an E1a-like activity within the cell. The tenfold increase in the titre of *dl*312 represents an HMBA-mediated alteration of gene expression of a non-herpesvirus gene in its natural environment, implying a more universal mode of action.

As HMBA-treated HeLa nuclear extracts did not promote complex formation at the TAATGARAT element, subsequent investigations concentrated on the binding of transcription factors at other promoter motifs, namely the Ad MLP, CCAAT box and Vmw110 TATA box. Analysis of complex formation at the TATA box is relevant as an acidic activator, such as Vmw65, stimulates transcription by increasing the number of functional preinitiation complexes by recruiting TFIID to the promoter (Lin & Green, 1991). However, use of HMBA-treated nuclear extracts, instead of untreated extacts did not promote complex formation at the Ad MLP, CCAAT box or Vmw110 TATA box. As HMBA does not act by inducing stable cellular alterations, gel retardation assays were performed using extracts exposed to HMBA for 1, 15, 30, 45 and 60min to ensure that any potential alterations are not missed. Even with only short exposures, HMBA treatment did not increase complex formation at any of the motifs analysed. Instead, there appeared to be a slight reduction in complex formation using HMBA-treated extracts, which is most pronounced for CCAAT box binding. This may reflect the modification or inactivation of factors involved in complex binding by the action of HMBA.

5.3 Sequence specificity

The question of sequence specificity in HMBA's mode of action was addressed using a number of plasmids with the CAT gene under the control of various viral promoters (Section 4.2.1). However, all the promoters tested to date shows increased CAT activity in the presence of 5mM HMBA. Therefore, in this transient system, exposure to HMBA increases the level of CAT activity from IE, E and L HSV promoters, and from the HCMV and SV40 enhancers. Campbell *et al.* (1990) also demonstrated that the transcription of many transfected genes is stimulated by HMBA, whereas both the Moloney murine sarcoma virus and mouse mammary tumour virus LTRs failed to show significant increases in CAT activity in the presence of HMBA. As a consequence of this apparent lack of correlation between promoter specificity and inducibility, Campbell *et al.* (1990) suggested that CAT activity may be regulated by the post-translational modification of one or more factors directly involved in transcription initiation. However, this

hypothesis does not explain why a particular gene that is responsive in both the viral genome and in plasmid-based assays, is no longer responsive to HMBA once integrated into the cellular genome. Run-on transcription assays need to be performed to determine whether HMBA increases the rate of transcription of IE RNAs, rather than their accumulation.

This question of specificity was subsequently addressed using a number of mutant viruses which, in contrast with the plasmid based CAT assays, allow the direct investigation of the effect of HMBA on different promoters in their native genomic environment.

The Moloney murine leukemia virus enhancer and promoter is not efficiently utilized in HFL cells (C.M. Preston, unpublished results). When this element is substituted for the Vmw110 promoter in the viral genome it is not responsive to activation by HMBA. In contrast, titration of mutants *in*1827 and *in*1833, in the presence and absence of HMBA, reveals that the HCMV enhancer and SV40 promoter/enhancers respectively are responsive to activation when in the viral genome. Therefore, there appears to be a limited degree of specificity in the action of HMBA on genes in the viral genome. HMBA can act to increase the level of expression of the Vmw110 gene controlled by the IE-1 promoter, but cannot switch on its expression when under the control of the non-responsive Moloney murine leukemia virus enhancer. It is possible, however, that under IE conditions *in*1827 does express Vmw110 mRNA at a level not detectable in dot blots which is increased to a still undetectable level in the presence of HMBA.

This inability to demonstrate increased activity using HMBA-treated extracts in an *in vitro* transcription initiation system may be explained by the fact that HMBA does not increase the titre of *in*1814 by inducing stable cellular alterations. Pretreatment of cells with HMBA prior to infection with *in*1814 has no effect on the resulting titre. In this system, no significant differences were detected in transcriptional activity using untreated and HMBA-treated extracts, from the Vmw110 promoter, Vmw68 promoter in the presence and absence of upstream sequences, from the Ad MLP or the HCMV IE promoter. However, these results do not preclude the possibility that specific factors affecting the transcriptional activity of an extract are modified in response to HMBA treatment, but are not detected as a consequence of their unstable nature.

The C-terminal domain (CTD) of the large subunit of RNA polymerase II, containing multiple repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Allison *et al.*, 1985; Corden *et al.*, 1985), is a potential cellular target for HMBA-directed post-translational modification. This essential

structural feature is unique to RNA polymerase II and is modified by phosphorylation in a post-translational process (Breant *et al.*, 1983). Immunoprecipitations can be performed to compare the electrophoretic mobilities of RNA polymerase II isolated from untreated and HMBA-treated HeLa cells. Any variations in mobility in SDS-PAGE may be attributable to different degrees of phosphorylation at the hydroxyl-rich CTD. Although the *in vitro* transcription experiments (Section 4.5) did not reveal any significant difference in transcriptional activity between untreated and HMBA-treated extracts, it is possible that unstable modifications which increase the activity of the enzyme have not been detected, as the CTD is highly susceptible to proteolysis (Sentenac, 1985).

The polar planar molecule, HMBA, does not appear to function directly as a consequence of its overall negative charge with a mechanism similar to that of an acidic activator, as the inclusion of HMBA in the transcription reactions does not increase the amount of RNA produced from RNA polmerase II type promoters. In addition, hypoxanthine, which is not strongly charged, also significantly increases the titre of *in*1814. The effect of HMBA on transcription catalysed by RNA polymerase III was analysed using the Ad VA promoter. Again, neither HMBA-treatment of extracts nor the inclusion of HMBA in the assays increased transcriptional activity. The effect of HMBA exposure on transcription from an RNA polymerase III promoter in a transfection assay has not yet been investigated. As all the RNA polymerase type II promoters analysed in transfection assays to date have shown increased activity in the presence of HMBA, then a degree of promoter specificity may be established if RNA polymerase III promoters fail to respond in transfection assays.

5.4 Importance of chromatin structure

During the establishment of a latent infection, Harris and Preston (1991) demonstrated that the *in*1814 genome rapidly becomes refractory to activation by Vmw65. Once latency has been established, *in*1814 cannot be reactivated or complemented by Vmw65, suggesting that the IE promoters become insensitive to transactivators, with the exception of Vmw110 (Harris *et al.*, 1989; Harris & Preston, 1991). It is thought that this lack of response may represent an overall silencing of the *in*1814 genome as a result of chromatin formation (Deshmane & Fraser, 1989) or the presence of a repressor molecule which prevents the expression of IE genes and hence prevents reactivation (Kemp, 1990). As a similar refractoryness is observed

with time during *in*1814 lytic infection in the presence of HMBA, it is possible that this too is an effect of the overall silencing of the genome. Within 5h of infection, the *in*1814 genome is converted to a non-linear configuration (Harris & Preston, 1991), and it is feasible that this phenomenon also interferes with the ability of HMBA to complement *in*1814. In other words, as the population of viral genomes gradually moves towards a non-linear configuration, this alteration in structure may preclude HMBAs action.

The importance of chromatin structure in the ability of a promoter to respond to activation by HMBA is highlighted by the observation that the IE-3 promoter is responsive to HMBA both in a plasmid based transfection assay and in the viral genome, but is unresponsive once integrated into the cellular genome. The transformed cell line, 143TKN, containing the TK gene under the control of the HSV-1 IE-3 promoter, is not responsive to activation by HMBA, despite these regulatory sequences retaining responsiveness to the viral transactivator Vmw65. Similarly, expression from the IE-4 promoter was found to be activated by HMBA treatment during *in*1814 infection with the accumulation of IE-4 RNA, yet TK assays reveal that the IE-4 promoter is not responsive to HMBA once integrated into the cellular genome. Despite this non-responsiveness to HMBA, the IE-4 promoter in 143TKTaqA cells is in a structural conformation which is accessible to viral transactivators as infection with UV-irradiated *ts*K, which provides Vmw65, significantly increased TK activity. Transfection assays, in the presence and absence of HMBA, also reveal that the early TK promoter is responsive to HMBA in this plasmid based assay. However, TK assays with 143TK1 cell extracts reveal that the TK promoter when in the cellular genome is not activated by exposure to HMBA, despite retaining the ability to be transactivated by Vmw110.

Therefore, there appears to be a feature of the cellular genome's environment which impedes activation by HMBA, without otherwise affecting the responsiveness of the IE-3, IE-4 and TK promoters to activation by the appropriate viral transactivator. Work needs to be carried out to determine the structural aspects of promoter DNA in plasmids and in the viral genome which facilitates activation by HMBA. The early stimulation of CAT activity in transient transfectants and increased IE RNA transcription during *in*1814 infection, is not sufficient to increase expression from the same promoters once integrated into the cellular genome. There is a delay of 12-24h following exposure to inducer before the induction of mouse-globin genes occurs (Salditt-Georgieff *et al.*, 1984; Sheffery *et al.*, 1984). This delayed

response may reflect a requirement for *de novo* protein synthesis of transcription factors, or be a consequence of chromosomal integration.

The incorporation of DNA into a chromatin structure has been shown to inhibit the access of transcription factors (Emerson & Felsenfeld, 1984; Workman & Roeder, 1987; Workman *et al.*, 1988; Cheng & Kelly, 1989). In the stable TK^+ transformed cell lines, 143TK1, 143TKN and 143TKtaqA, the introduced DNA is in chromatin structure which, although accessible to either Vmw65 or Vmw110, does not appear to be responsive to HMBA. The fact that the transformed cell lines are responsive to these viral transactivators but not HMBA, suggests that the effect of HMBA is on one or more factors which are not involved in transactivation by Vmw65 or Vmw110. It is possible that activation of these stably integrated genes may depend on increased access to the promoters of a factor, modified by the action of HMBA, as a result of cell-cycle events (Conkie et al., 1981), changes associated with DNA replication (Enver *et al.*, 1988) or alterations in the state of DNA methylation (Razin *et al.*, 1980). In contrast, both plasmid DNA, introduced by transient tranfection, and viral DNA, are initially naked, providing an environment in which the control regions are not associated with histones and are therefore freely accessible to transcription factors.

Becker *et al.* (1991) demonstrated that the simultaneous addition of TFIID and histones during chromatin assembly on the *Drosophilla* hsp70 promoter resulted in the formation of 'potentiated' chromatin, capable of responding to the subsequent addition of heat shock transcription factors. It is possible that HMBA may function in an analogous manner, activating transcription from IE genes when in plasmids or in the viral genome as a result of the open active chromatin structures.

Sodium butyrate is a known inducer of MELC differentiation (Leder & Leder, 1975) and is associated with the hyperacetylation of histones H3 and H4 through the inhibition of histone deacetylation (Candido *et al.*, 1978) and the accumulation of a non-histone chromatin protein IP_{25} (Keppel *et al.*, 1977). However, as other inducers such as DMSO do not cause hyperacetylation of histones, it is unlikely that butyrate induced hyperacetylation of H3 and H4 in MELCs has any direct relationship to the induction of differentiation by this agent. Sodium butyrate does not increase the titre of *in*1814 in HFL or BHK cells (C.M. Preston, personal communication). The PKCε fraction isolated from MELCs has the capacity to phosphorylate histones. In contrast, the induction of IP_{25} by sodium butyrate treatment also occurs in response to a large number of chemical differentiation inducing agents (Candido *et al.*, 1978).

Another link between induction of MELC differentiation and HSV gene expression was made when Kemp and Latchman (1989) demonstrated that treatment of neuronal C1300 cells with sodium butyrate reverses a transcriptional block, and thereby facilitates the expression of HSV IE genes. In addition, Ash (1986) demonstrated that exposure of C1300 cells to sodium butyrate increases the permissivity of these cells for HSV infection. The effect of sodium butyrate treatment of C1300 cells on HSV IE gene expression is therefore unlikely to involve the same mechanism that is responsible for the HMBA-mediated increase in *in*1814 IE gene expression.

Acetylation of histones can affect the chromatin structure of a gene, being associated with increased sensitivity to DNaseI and micrococcal nuclease, and hence with transcriptionally poised chromatin. The 143TK1, 143TKN and 143TaqA transformed cell lines could be treated with sodium butyrate and TK assays performed to determine whether the action of this compound results in increased TK activity, either alone or in conjunction with HMBA. Similarly, the transformed cells could be treated with 5-azacytidine, in the presence and absence of HMBA, to determine whether demethylation has any effect on the ability of HMBA to activate expression of these selected genes integrated into the cellular genome. Before these experiments are carried out the 143TK1, 143TKN and 143TaqA transformed cell lines should be cloned and characterised.

5.5 Role of HMBA during reactivation of latent virus

During latency *in vivo*, Vmw65 is not thought to be present, thus reactivation must occur through a pathway that does not initially require this protein. Infection with *in*1814 provides a situation in which Vmw65 activity is absent or severely reduced, suggesting the stimulation of IE RNA levels observed in tissue culture cells represents the mechanism by which HMBA and DMSO enhance reactivation from the ganglia (Whitby *et al.*, 1987; Bernstein & Kappes, 1988; Leib *et al.*, 1989).

It has been recently reported that HMBA enhances the replication of HSV in IMR-32 human neuroblastoma and HEp-2 cells after infection at low m.o.i. (Yura *et al.*, 1991), and that HMBA improves 'spontaneous' reactivation of latent HSV-2 from infected IMR-32 cells (Kondo *et al.*, 1990). From the studies presented in this thesis, it is likely that these events are due to stimulation of IE gene expression by HMBA.

Determination of the molecular basis for the activity of HMBA on *in*1814 infection may reveal the primary events by which the compound

affects the poorly understood process of reactivation. Alternatively, the response to HMBA may operate after the initial trigger for reactivation, simply by accelerating virus growth in explanted ganglia. The fact that the stimulation of reactivation of an HSV-1 mutant deleted for Vmw110 coding sequences is "all or nothing" (Leib *et al.*, 1989) supports the former pathway, but it is possible that the results could be accounted for by the relatively small effect on replication of mutants that do not produce Vmw110 (namely *dl*1403 and *in*1825).

Since the action of HMBA on *in*1814 is not thought to be due to demethylation but an effect on IE gene expression, it follows that the enhancement of reactivation may be due to the same activity. I would therefore favour the view that HMBA and DMSO improve reactivation of latent HSV through direct modulation of viral or cellular gene expression by a mechanism related to their effects on MELCs and distinct from the action of 5-azacytidine, assuming that 5-azacytidine primarily acts by demethylation.

Sears *et al.* (1991) previously showed that the neuronal expression of Vmw65 did not preclude the establishment of a latent infection. By investigating whether latency can be established *in vitro* in the presence of HMBA, one can confirm that, as expected, increased IE gene expression precludes the establishment of an *in*1814 latent infection. In addition, the *in vitro* system developed by Russell and Preston (1986) could be employed to determine whether exposure to HMBA prevents HSV-2 establishing latency *in vitro* at the supraoptimal temperature, 42°C. As HMBA functions through a mechanism whereby IE gene expression is increased, it is reasonable to hypothesize that exposure to HMBA would tip the lytic-latent decision in favour of a lytic infection. The mutant *in*1820, in which the Vmw110 promoter in *in*1814 has been replaced by the murine Moloney leukaemia virus enhancer, expresses Vmw110 poorly in HFL cells and does not respond fully to HMBA. Experiments could be carried out to determine what effect HMBA-treatment has on the ability of *in*1820 to establish latency *in vitro*.

The cAMP responsive element (CRE) in the LAT promoter may be involved in modulation of LAT promoter expression and the control of viral reactivation (Leib *et al.*, 1991) as compounds which increase the intracellular levels of cAMP also promote the reactivation of latent virus from the ganglia (Saiz de la Meza *et al.*, 1989; Foster *et al.*, 1989). Prostoglandins are examples of experimental reactivation inducing stimuli which are associated with increased cAMP concentrations (Kimberg *et al.*, 1972; Matsuzawa & Nurenberg, 1975; Rodbell, 1980; Lentzki, 1988; Hu & Gudas, 1990). It is feasible that exposure to HMBA alters LAT expression through this control element, as

HMBA-induced MELC differentiation is also associated with a transient rise in the concentration of cAMP. Gel retardation assays may be performed in order to compare complex formation at the CRE in conjunction with untreated and HMBA-treated extract nuclear extracts. As the prostoglandin syhthesis inhibitor, indomethacin, reversibly inhibits reactivation of latent HSV from ganglia (Blyth *et al.*, 1976; Harbour *et al.*, 1983; Kurane *et al.*, 1984), titration could be performed to determine whether this compound also antagonizes the HMBA-mediated rise in *in*1814 titre. If so, then it is likely that the mechanism of HMBA-mediated reactivation of latent virus involves the modulation of intracellular levels of cAMP.

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