INVESTIGATION OF THE MECHANISMS OF

TRANSCRIPTION TRANSACTIVATION

BY THE HERPES SIMPLEX VIRUS PROTEIN VMW65

by

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree of Doctor of Philosophy

McMaster University,

THE HERPES SIMPLEX VIRUS TRANSACTIVATOR VMW65

DOCTOR OF PHILOSOPHY (1993)   McMASTER UNIVERSITY
(Biochemistry)   Hamilton, Ontario

TITLE: Investigation of the Mechanism of Transcription
            Transactivation by the Herpes Simplex Virus
            Protein Vmw65

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NUMBER OF PAGES: xxi,237

ABSTRACT

Eukaryotic gene expression is regulated through the assembly of transcription factors into multi-component complexes that interact with cis-acting DNA recognition sites. The herpes simplex virus protein, Vmw65 (also known as VP16, αTIF), stimulates transcription through direct and indirect contacts with specific cellular factors (including the ubiquitous octamer binding protein, Oct-1) and a conserved enhancer-like DNA sequence (consensus TAATGARAT) found upstream of the viral immediate-early (IE) genes. The objective of this project was to utilize this viral system as a model to investigate mechanisms by which eukaryotic gene transcription is regulated. The functional interactions of Vmw65 with the cellular transcription machinery make this a valuable system for the elucidation of the protein-protein and protein-DNA interactions involved in regulation. This thesis describes various strategies that have been utilized to examine the different aspects Vmw65 mediated transactivation.

The structure-function profile of Vmw65 was determined through the construction of a series of mutants that were individually assayed for specific activities. An *in vivo* transient transfection system was utilized to measure the

ability of each of the mutants to transactivate an indicator gene, containing a TAATGARAT sequence, relative to the wild type protein. This assay was also utilized to map domains of Vmw65 that are capable of interfering with the wild type transactivation function in a trans-dominant manner. Finally, the mutants were expressed in *E. coli* as protein A fusion proteins and each was analyzed for its ability to direct the assembly of the Vmw65 dependent complex and to bind directly to DNA in the absence of other factors.

To summarize, the transactivation function is highly sensitive to mutations within two distinct internal regions of the protein (corresponding to amino acids 178-215 and 335-379). A region of Vmw65, located between amino acids 141 and 186, is capable of interfering with the wild type transactivation function *in vivo* and is required for complex assembly *in vitro*. These results suggest that this domain forms essential contacts with a factor involved in the transactivation function of Vmw65. The transcription activation domain of Vmw65 is not required for complex assembly indicating that Vmw65 is a modular protein consisting of a transcription activation domain and a complex assembly domain. Each of these domains can independently impart its activity to a heterologous protein.

Additional cellular components of the Vmw65 dependent complex were identified and characterized using cell

fractionation techniques. One factor, designated SF, was shown to specifically stabilize the multi-protein complex. SF has an apparent molecular weight of 1500-3000 Da and is resistant to heat treatment as well as extensive digestions with protease, nuclease and phospholipase. ß-glucuronidase digestion did have an effect on SF activity suggesting that this factor may be composed, at least partially, of carbohydrate. This factor could be an adapter molecule that facilitates the interaction of activator proteins such as Vmw65 with specific DNA binding factors such as Oct-1.

Finally, the role of Vmw65 in the herpes simplex virus lytic cycle was investigated through the construction and characterization of a stable cell line (designated BSV65) expressing this transactivator. The cell line was shown to specifically activate viral immediate early genes and was capable of complementing a virus defective for the Vmw65 transactivation function. The transfection of purified HSV-1 DNA into BSV65 cells resulted in a 200 fold increase in virus production relative to the parental cell line. This result suggests that the low specific activity of HSV-1 DNA can, at least partially, be explained by the absence of this specific IE gene transactivator. The availability of the BSV65 line has facilitated the study of the physiological role of Vmw65 protein in the course of an HSV-1 lytic infection.

LIST OF PUBLICATIONS

The studies presented in this thesis have been reported, in part, in the following publications:

1.   Werstuck, G. and Capone, J.P. (1989). Mutational analysis of the herpes simplex virus trans-inducing factor Vmw65. Gene 75, 213-224.

2.   Werstuck, G. and Capone, J.P. (1989). Identification of a domain of the herpes simplex virus trans-activator Vmw65 required for protein-DNA complex formation through the use of protein A fusion proteins. J. Virol. 63, 5509-5513.

3.   Werstuck, G., Bilan, P. and Capone, J.P. (1990). Enhanced infectivity of herpes simplex virus type 1 viral DNA in a cell line expressing the trans-inducing factor Vmw65. J. Virol. 64, 984-991.

4.   Capone, J.P. and Werstuck, G. (1990). Synthesis of the herpes simplex transactivator Vmw65 in insect cells using a baculovirus vector. Mol. Cell. Biochem. 94, 45-52.

5.   Werstuck, G. and Capone, J.P. (1993). An unusual cellular factor potentiates protein/DNA complex assembly between Oct-1 and Vmw65. J. Biol. Chem. (in press).

6.   Xiao, P., Werstuck, G. and Capone, J.P. Analysis of the requirements for DNA binding and protein interactions of the HSV-1 trans-activator Vmw65 for multi-component protein/DNA complex assembly. (in preparation).

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. John Capone, for his support, input and enthusiasm during the course of this work. Thanks are also extended to the members of my committee; Dr. H.P. Ghosh, Dr. John Hassell and Dr. Jim Smiley.

I would also like to extend my appreciation to my many good friends within and outside the Department of Biochemistry for making the time I have spent here very enjoyable.

Special thanks to my family, and especially my wife, Michele, whose love and encouragement I could always count on, and Connor, for those lengthy discussions that never failed to keep things in perspective.

Finally, I would like to acknowledge the National Cancer Institute of Canada for their financial support.

TECHNICAL ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

A	Ampere
aa	amino acid
AAD	acidic activation domain
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CFF	complex formation factor
Ci	Curie(s)
CMV	Cytomegalovirus
CoA	coenzyme A
cpm	counts per minute
CTD	carboxy-terminal domain
Da	dalton
ddH$_2$O	deionized distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	early
EDTA	ethylenediaminetetracetic acid

g	gram(s)
GA⁻	GARAT minus
g_{av}	acceleration of gravity
GABP	GA Binding Protein
GTP	guanosine triphosphate
HCF	host cell factor
HEPES	n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
hpi	hours post infection
HSV-1	Herpes Simplex Virus type 1
ICP	infected cell protein
IE	immediate early
IgG	immunoglobin G
Inr	initiator
IPTG	isopropyl ß-D-thiogalactoside
k	kilo
kb	kilo base pair
L	late
LAT	latency associated transcripts
μ	micro
m	milli
M	molar
min	minute(s)
MOI	multiplicity of infection
mol	mole(s)

mRNA	messenger ribonucleic acid
MW	molecular weight
n	nano
NBT	nitroblue tetrazolium
NF	nuclear factor
NMR	nuclear magnetic resonance
NP40	Nonidet P-40
NTP	nucleoside 5'-triphosphate
OBP	octamer binding protein
oc⁻	octamer minus
OD_x	optical density at wavelength x
ORF	open reading frame
OTF-1	octamer binding transcription factor
p	pico
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PFU	plaque forming unit(s)
PMSF	phenylmethylsulfonyl flouride
pol	polymerase
PRV	Psuedorabies Virus
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate

SF	stimulatory factor
snRNA	small nuclear ribonucleic acid
TAF	TBP associated factor
TBP	TATA-box binding protein
TBE	Tris-borate EDTA buffer
TBST	Tris-borate saline plus Tween 20
TE	10 mM Tris-HCl, pH 7.5, 1 mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	transcription factor
TK	thymidine kinase
TRF	TAATGARAT recognition factor
Tris	Tris(hydroxymethyl)aminoethane
tRNA	transfer ribonucleic acid
ts	temperature-sensitive
UAS	upstream activating sequence
UL	unique long
US	unique short
UV	ultra-violet
V	volt(s)
VCAF-1	Vmw65 complex assembly factor
VIC	Vmw65 induced complex
VP	virion protein
VZV	Varicella Zoster Virus
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

INTRODUCTION

The many structural and catalytic functions required for normal cell growth and development are dependent upon the synthesis of genomically encoded RNA and protein molecules. Specific gene products are required at different times and in widely differing quantities as a cell progresses through its growth/division cycle and makes the appropriate responses to various physiological stimuli. This requirement, together with the huge number of genes involved in the operation of a cell (there are an estimated 100 000 genes in a human diploid cell) make it essential that expression be tightly controlled. Aberrations in a cell's gene expression programming can drastically effect the characteristics of the cell and have devastating consequences for the organism of which it is part.

The activity of an individual gene product can be regulated at many levels including splicing (Smith et al., 1989), translation (Hersey, 1991) and by post-translational modification (Hunter and Karin, 1992). However, it is apparent that, in the majority of cases, the principle control is exerted during the transcription initiation step. There would appear to be an economical advantage for locating the control step early, rather than late, in the process. In this manner,

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the energy expended on the expression of unneeded gene products is minimized.

Eukaryotic genes can be divided into three classes by virtue of the polymerase that is responsible for the catalysis of transcription. RNA pol I synthesizes large transcripts that are processed into ribosomal RNA within the nucleolus (Reeder, 1990). RNA pol III synthesizes small RNAs and transfer RNA precursors (Geiduschek and Tocchini-Valentini, 1988). Both class I and III genes are generally thought to represent constitutive, "house keeping" functions of the cell, although there are examples of regulated expression (Bredow et al., 1990; Lorch et al., 1990). Most, if not all, genes that encode functional proteins are transcribed by RNA pol II. Therefore RNA pol II transcription initiation represents a major control point in the many cellular pathways that rely upon protein synthesis.

1.1   The Initiation of RNA Polymerase II Transcription

Transcription factors involved in the expression of class II genes can be broadly separated into two categories; those required for basal expression, and those that modulate (both positively and negatively) the level of transcription. The basal transcription machinery consists of at least 7

different and distinct activities (designated TFIIA, IIB, IID, IIE, IIF, IIH and IIJ). Each of these factors are composed of one or more peptides (see Table 1.1.1, reviewed in Zawel and Reinberg, 1992). These factors assemble in an orderly fashion with RNA pol II to form a transcription pre-initiation complex just upstream of the transcription start site (Buratowski et al., 1989). All of the general factors are not necessarily needed for the initiation of basal level transcription. The requirement for at least some of the factors (for example TFIIA, IIH, IIJ) is promoter dependent (Parvin et al., 1992).

The basal promoter, sufficient to support the formation of the pre-initiation complex (see below), consists of an initiator sequence (INR) which encompasses the transcription start site (Smale and Baltimore, 1989) and, in most cases, a TATA box located (in higher eukaryotes) 25-30 bases upstream of the transcription start (Matsui et al., 1980). The sequences of the TATA motif and the INR element are widely variable between different genes (Zawel and Reinberg, 1992). In addition, some genes appear to contain TATA-less promoters. The individual characteristics of a promoter may impart differences in the basal level activities and/or could be involved in the regulation of gene expression (Smale and Baltimore, 1989).

The initial step in transcription involves the recognition of the TATA sequence by TFIID. TFIID is the only

FACTOR	PEPTIDE COMPOSITION	NATIVE MASS	ROLE IN PIC FORMATION
TFIID	38kDa(TBP) + TAFs	>100kDa	TATA-box recognition co-activators of transcription
TFIIA	34kDa 19kDa 14kDa	?	stabilizes TFIID/TATA complex
TFIIB	33kDa	33kDa	binds to DA/TATA complex
TFIIF	30kDa 74kDa	220kDa	binds to (recruits?) RNA pol II
TFIIE	34kDa 56kDa	200kDa	binds to DApolF complex
TFIIH	90kDa 62kDa 43kDa 41kDa 35kDa	230kDa	CTD kinase involved in transcription initiation
TFIII	120kDa[a]	120kDa[a]	Inr recognition
TFIIJ	?	?	binds to DApolFEH complex
pol II	at least 10-12 peptides[b]	>500kDa	template directed RNA synthesis

Table 1.1.1   The general transcription factors of the pre-initiation complex. Listed are the general factors identified to date as well as their peptide compositions. Adapted from Zawel and Reinberg (1992). [a] from Roy et al. (1991). [b] from Sawadogo and Sentenac, 1990).

basal transcription factor to have a sequence specific DNA binding activity and therefore is responsible for positioning the entire transcription complex on the gene (Parker and Topol, 1984). TFIIA then binds to and stabilizes the TFIID-TATA complex, forming what is known as the template committed complex (Sawadogo and Sentenac, 1990). The remaining factors assemble around TFIID in the following order; TFIIB, the pol II/TFIIF complex, TFIIE, TFIIH, and finally TFIIJ (Fig 1.1.1A, reviewed in Roeder, 1991; Zawel and Reinberg, 1992). The transcription pre-initiation complex is thought to assemble via a different pathway on promoters lacking a TATA box (Carcamo et al., 1991; Colgan and Manley, 1992) and may require an additional factor, TFII-I (Roy et al., 1991), although there remains a requirement for TFIID (Pugh et al., 1991; Wiley et al., 1992). TFIID consists of a core protein, the TATA-binding protein (TBP) of 38 kDa, that is tightly bound to factors that have been designated TAFs (TBP associated factors, Berger et al., 1990; Kelleher et al., 1990) or TIFs (transcription intermediary factors, White et al., 1991; reviewed in Gill and Tjian, 1992). At least 6 TAFs have been identified to date (Tanese et al., 1991; Timmers et al., 1992). Recombinant TBP is capable of driving basal transcription just as well as the TFIID holo-complex however, TBP on its own cannot support activated transcription. Subsequently at least some of the TAFs have been designated
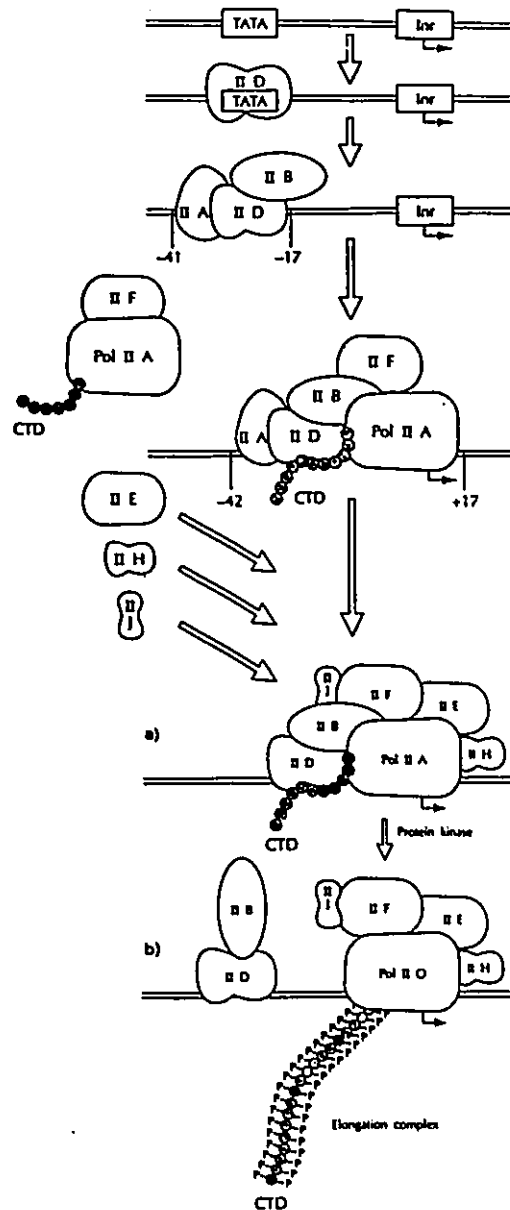
Fig. 1.1.1 **a) A schematic diagram of the transcription pre-initiation complex** showing the general transcription factors identified to date and the order in which they are believed to assemble. The positioning of each factor in the complex is based upon DNAse I protection and protein-protein binding assays.
b) The initiation of transcription is believed to be triggered by a protein kinase that specifically phosphorylates the CTD of the large subunit of RNA pol II. This reaction stimulates the dissociation of pol II from the templated committed complex (TFIID/A). Adapted from Zawel and Reinberg (1992).

co-activators (Dynlacht et al., 1991). It is becoming increasing clear that the transcription of class I, II and III genes have at least one factor, TFIID, in common (Cormack and Struhl, 1992; Schulz et al.,1992; Sharp, 1992). The TAFs are believed to play a role in promoter specificity since the TFIID activity required for RNA pol I and II mediated transcription is contained within distinct complexes (Comai et al., 1992).

RNA pol II is a large, complex, multi-subunit enzyme containing at least 8-12 different peptides, many of which have yet to be cloned (reviewed in Sawadogo and Sentenac, 1990; Young, 1991). The largest of these subunits contains a carboxyl-terminal domain (CTD) consisting of multiple, tandem repeats of the sequence Tyr Ser Pro Thr Ser Pro Ser. The differential phosphorylation of this repeat domain is thought to play an important role in the transcription reaction (Zehring et al., 1988) . When CTD is non-phosphorylated the pol II enzyme can assemble into a pre-initiation complex (Lu et al., 1991) perhaps by specifically binding to TFIID (Koleske et al., 1992). RNA pol II containing a highly phosphorylated CTD has been correlated with transcription elongation (Payne et al., 1989) and appears to be deficient in binding TFIID (Usheva et al., 1992). Specific CTD kinase activity has been detected in cell extracts (Arias et al., 1991; Feaver et al., 1991). TFIIH and its yeast homologue,

BTF-2, have recently been shown to contain a kinase activity that specifically phosphorylates the CTD of RNA pol II (Gileadi et al., 1992; Lu et al., 1992). The current model of transcription initiation (Fig. 1.1.1B) is that RNA pol II assembles into the pre-initiation complex with the CTD in the non-phosphorylated form. The phosphorylation of CTD drives the dissociation of RNA pol II from TFIID, releases the transcription machinery from the start site and allows transcription elongation to proceed. The process is then repeated as a new pre-initiation complex forms on the committed template (Laybourn and Dahmus, 1990).

The factors and mechanisms involved in basal transcription have been intensely studied and much has been learned in the past few years. The delineation of the individual steps in the assembly of the pre-initiation complex and transcription initiation have revealed many stages where regulators of transcription may act (Fig. 1.2.1). Subsequently these interactions have become a major area of research.

1.2   The Regulation of Transcription

The system that has evolved to fulfil the formidable requirements of gene regulation (reviewed in Johnson and McKnight, 1989) makes use of numerous conserved, cis-acting
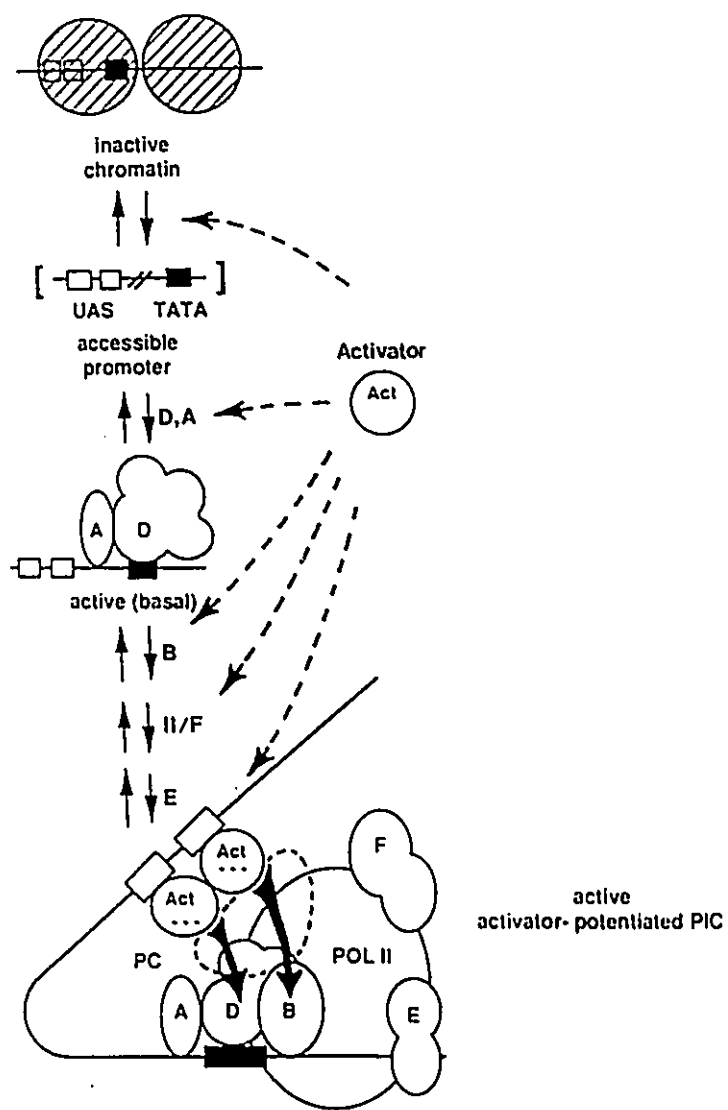
Fig. 1.2.1   A model of transcription activation.
Activators can potentially work at several different stages
prior to the initiation of transcription (indicated by the
dashed arrows). These include; i) the depression of nucleosome
bound promoters, ii) the formation or stabilization of
committed templates, or iii) in the recruitment of one or more
of the other general transcription factors into the pre-
initiation complex. The interaction of the regulatory factors
with upstream recognition motifs confer specificity to the
activation function. This figure shows two transactivators
interacting directly, or through positive cofactors (PC) with
TFIID and TFIIB of the pre-initiation complex. Transcription
may also be regulated during the elongation and/or termination
steps. Adapted from Roeder (1991).

DNA sequences located upstream of the transcription start site. These have been termed Upstream Activating Sequences (UAS) in yeast (Guarente et al., 1982) and enhancers in higher eukaryotes (Banerji et al., 1981). Individual genes contain different numbers and combinations of these recognition sequences thereby conferring specific and unique expression patterns for each gene. Transcription is regulated through the cis-acting sites by the appropriate trans-acting factors (Dynan, 1989). The transcription regulatory factors generally contain combinations of conserved, modular domains that allow them to bind to specific DNA sequences and interact with other transcription factors (Brent and Ptashne, 1985; Frankel and Kim, 1991). Thus, the modulation of transcription of a particular gene requires both the specific recognition of a cis-acting DNA sequence(s) and the appropriate interaction(s) between the (DNA bound) trans-acting factor(s) and the transcription initiation machinery. This is believed to occur though the looping out of the intervening DNA sequences (Ptashne, 1988). The large number of possible combinatorial interactions allow the genes of the eukaryotic genome to be controlled specifically though protein-protein and protein-DNA interactions without the system becoming unmanageably complex. These regulators can be repressors (Levine and Manley, 1989) and/or activators of transcription (Ptashne, 1988; Lillie and Green, 1989a).

There are four families of DNA binding domains that are commonly found among eukaryotic transcription factors (reviewed in Harrison, 1991). The members of each family are capable of specifically recognizing a wide variety of DNA sequences even though the overall conformation of the motif is conserved. This is possible though specific differences in the amino acids that make intimate contact with the bases of a recognition sequence. The helix-turn-helix domain, found in over 80 known proteins from yeast to man, is similar in many ways to the helix-turn helix DNA binding domain first described in prokaryotes (Laughon and Scott, 1984). This structure, referred to in some cases as a homeodomain, has been solved using two-dimensional NMR (Otting et al., 1990) and X-ray crystallography (Kissinger et al., 1990). In a subset of helix-turn-helix DNA binding proteins the motif is extended into what is now known as a POU domain (for Pit-1, Oct-1, unc-86, three proteins that share this region, Sturm et al., 1988; Herr et al., 1988). The leucine zipper basic region (bZip), present in GCN4, C/EBP, fos, myc, jun plays a role in dimer formation as well as DNA binding (Vinson et al., 1989). The GCN4 structure has recently been elucidated by X-ray crystallography (Rasmussen et al., 1991). Helix-loop-helix DNA binding domains are found in the glucocorticoid and estrogen receptors. These structures have been solved by two-dimensional NMR (Schwabe et al., 1990). The zinc finger domain

is present in TFIIA, SP1 and GAL4 and has been investigated using two-dimensional NMR (Lee et al., 1989) and X-ray crystallography (Pavletich and Pabo, 1991). In addition there are many DNA binding proteins which do not fit into any of the current categories. While the structure of at least one member of each of the DNA binding domain families has been solved these motifs are usually assigned to proteins by virtue of sequence homologies and computer modelling.

The study of many different transcription transactivators has led to the identification of three distinct activation motifs. The acidic activation domain (AAD), initially identified in the GAL4 and GCN4 contains a high density of negatively charged amino acids (Hope and Struhl, 1986; Ma and Ptashne, 1987a,b). The mechanism by which AADs operate has been conserved from yeast to mammals allowing activators from one organism to be fully functional in the other and vice versa (Ptashne, 1988; Cousens et al., 1989). Glutamine rich activation domains have been located in SP1 and Ubx (Courey and Tjian, 1988). Proline rich activation domains are found in CTF/NF1, jun and AP2 (Mermod et al., 1989). It is not yet clear how any of these domains are capable of modulating the enhancement of transcription although most models suggest interactions between the activation domain and some component of the initiation complex are required. Several models have been proposed to explain how an activation domain

may stimulate gene expression. First, the activation domain may assist in the recruitment of basal transcription factors or RNA pol II in the assembly of the pre-initiation complex. Similar interactions could be utilized to stabilize the pre-initiation complex (supported by the results of White *et al.*, 1992). Transactivators could also act by enhancing the number of pol II transcription initiation events from a preformed complex (reviewed in Lillie and Green, 1989a). Finally, an activator could operate by de-repressing the transcription of genes that are packaged in the form of chromatin (Croston et *al.*, 1991; Laybourn and Kadonaga, 1991). There is evidence that different transcription activators function by distinct mechanisms (Martin *et al.*, 1990; Tasset *et al.*, 1990). In addition, a single transactivation domain can be capable of utilizing more than one of the above strategies to enhance the expression of a particular gene (Laybourn and Kadonaga, 1991; Kuhn and Grummt, 1992).

At least two different approaches have been extensively utilized to determine how activators actually function to increase the transcription of a gene. The first involves determining which factors interact with the activator in the hope that their identity will shed light on the process itself. Thus far, the results of experiments involving AAD interactions have been conflicting. An AAD has, in separate but similar experiments, been shown to interact directly with

the TBP of TFIID (Horikoshi et al., 1991; Ingles et al., 1991) as well as with TFIIB (Lin et al., 1991). This same domain has also been implicated in binding of at least one TAF of the TFIID complex (Berger et al., 1990; Kelleher et al., 1990). To further complicate matters the AAD appears to interact with histone H1 in the derepression of transcription (Laybourn and Kadonaga, 1991). The second approach used to determine how activators function involves the elicitation of the physical structure of the domain in order to determine how it may be interacting with other components of the transcription machinery. Again most of this work has been concentrated on the AAD. The results of these experiments have provided no hard evidence for the existence of predicted structures (such as alpha helices, Zhu et al., 1990) under physiological conditions (Donaldson and Capone, 1992; O'Hare and Williams, 1992; Van Hoy et al., 1992). It is possible that a functional conformation is obtained only after contact is made with the proper ligand.

The study of the regulation of RNA pol II transcription necessitates the use of a model system that allows one to reliably recreate the phenomenon of interest under conditions where it can be measured and manipulated. Viral systems have been commonly used to study gene expression. This is largely due to the fact that viral transcription factors have been much easier to identify and

clone than their cellular counterparts. In addition, the viral genome is more easily manipulated than that of a diploid cell and the virus itself provides a convenient way to introduce foreign and synthetic genes into a cell.

1.3   The Herpes Simplex Virus as a Model for Transcription Regulation

The herpes simplex virus type 1 (HSV-1) possesses several characteristics that make it an excellent model system for the study of the regulation of RNA polymerase II transcription in eukaryotes. This DNA virus infects many established eukaryotic tissue culture cell lines. The entire 152 kb of HSV-1 genome has been sequenced and over half of the 70+ open reading frames (McGeoch et al., 1985, 1988) have been linked with proteins of known function (Honess and Roizman, 1973; Morse et al., 1978). Its short and relatively well defined lytic life cycle involves the highly regulated expression of the viral genes (Everett, 1987a; McKnight, 1987; Weinheimer and McKnight, 1987). Finally, the herpes simplex virus is dependent upon cellular RNA pol II as well as the basal transcription factors and many of the transcription regulator proteins provided by the infected host cell (Costanzo et al., 1977).

The >70 HSV genes can be organized into three

different categories according to the time post-infection that they are expressed (Fig. 1.3.1 A). These have been designated immediate-early (IE), early (E) and late (L) or, alternatively, α, δ and γ (Honess and Roizman, 1974). The E and L genes can be further subdivided into early (δ_1) and delayed-early (δ_2), leaky-late (γ_1) and true late (γ_2), respectively (Honess and Roizman, 1974; Costa et al., 1981). As suggested by the addition of the delayed-early and leaky-late gene classes, both of these systems of nomenclature are artificial, and in reality one cannot easily allocate the different genes into specific categories. All the genes of a particular category are not expressed with the same kinetics and the transition from immediate-early to late gene expression is, in most cases, gradual (Roizman and Sears, 1990 and references therein). For the sake of simplicity, and because this research is concentrated on only the earliest events in HSV gene regulation, the IE, E and L system of HSV gene classification will be used herein.

As previously stated, the distinction between the different classes of genes is temporal. IE transcripts begin accumulating just after infection and their levels peak at approximately 3 hours post infection (hpi) after which levels decline. There are at least 5 IE genes; designated IE0, IE4, IE22, IE27 and IE47 (encoding the infected cell proteins (ICP) 0, 4, 22, 27 and 47, respectively; Everett (1987a)). The IE

A
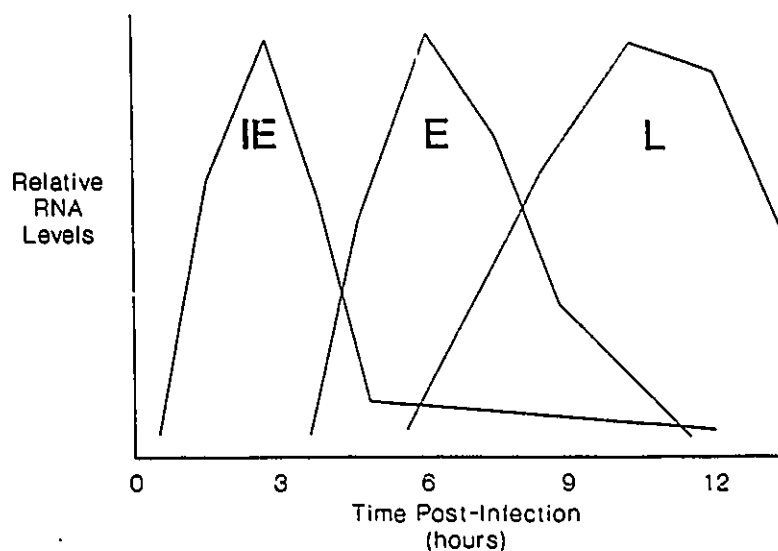
B
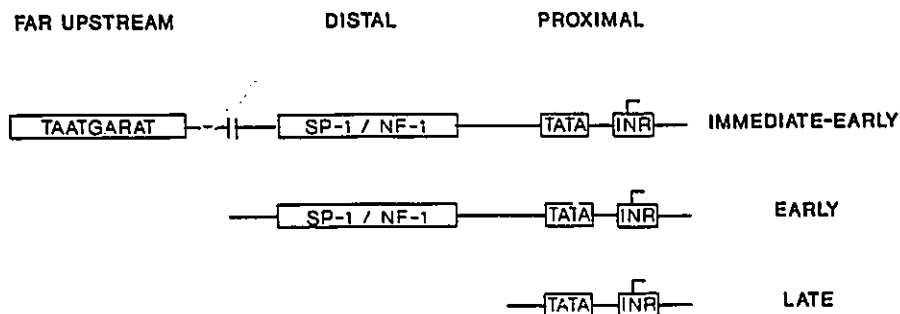
Fig. 1.3.1   A) The temporal control of HSV-1 gene expression. This figure illustrates the general shift from immediate-early to early to late gene transcription as the virus progresses through the lytic cycle of infection. The relative fluctuation in the levels of viral gene transcripts are plotted against time-post-infection. **B)** A schematic diagram showing the major control elements required for the regulated expression of the IE, E and L genes. Adapted from Everett (1987a).

proteins function as regulators of the cascade of HSV-1 gene expression. ICP0, 4 and 27 each have been implicated in the stimulation of E and/or L gene expression (Sacks et al., 1985,1987; Beard et al., 1986. ICP4 can negatively autoregulate its own transcription as well as that of other IE genes (O'Hare and Hayward, 1985b). Only ICP4 and ICP27 are essential for productive viral infection in tissue culture though the remaining IE genes may play various regulatory roles in the wild (Roizman and Sears, 1987). After receiving the appropriate stimulus from the IE gene products, the E transcripts accumulate up to 5-7 hours and then decrease. Examples of E proteins include, the 65 kDa DNA binding protein (UL42) and the viral DNA polymerase (UL30), both of which are required for viral DNA replication (Challberg, 1986). L transcripts are observed to accumulate for at least 12 hpi and encode many of the structural proteins required for virion assembly (reviewed in Roizman and Sears, 1987).

The cascade of HSV gene expression can be dissected with the use of chemical inhibitors or various temperature sensitive mutants which can arrest the virus at a particular stage as it progresses through its lytic life cycle. L gene expression, by definition, is dependent upon the completion of viral DNA replication. Thus cells infected with HSV in the presence of the inhibitors of DNA synthesis (cytosine arabinoside or hydroxyurea) exhibit reduced late protein

levels (Honess and Roizman, 1974), although, as always, there are exceptions (the leaky late genes). The reason behind the dependence of L gene expression on DNA replication is not yet clear.

The distinction between HSV IE and E/L genes is evident when cells are infected in the presence of the protein synthesis inhibitor, cycloheximide. Under these conditions E and L gene transcription is repressed while IE mRNA transcripts accumulate to high levels (Honess and Roizman, 1975). Thus E and L gene expression is dependent upon de novo IE protein synthesis whereas IE expression is reliant upon pre-existing cellular and/or viral factors.

The manner in which the three classes of viral genes are temporally controlled becomes evident upon the examination of their respective promoter/enhancer regions (Fig. 1.3.1 B). Each of the IE genes contain at least one copy of a conserved sequence (consensus TAATGARAT, R is usually a purine, Mackem and Roizman, 1982; Cordingley et al., 1983). This sequence is capable of conferring IE-like kinetics to a heterologous promoter (Preston et al., 1984; Gaffney et al., 1985). Typical of eukaryotic enhancer elements, the TAATGARAT motif operates independently of position and orientation in the IE promoter region (Lang et al., 1984; Preston and Tannahill, 1984).

1.4 Vmw65 is a Potent Stimulator of IE Gene Transcription

Early investigations into the kinetics of IE gene expression utilized a virus containing the IE4 promoter linked to an indicator gene (HSV-1 thymidine kinase (TK)). This synthetic gene (αTK) is regulated in a manner similar to the IE genes (Post et al., 1981). TK production in tissue culture cells transfected with this chimeric construct was greatly stimulated by HSV-1 infection with wild type virus, UV light-irradiated virus, or mutant virus defective in the release of the genomic DNA from the nucleocapsid (Batterson and Roizman, 1983). These results indicated that the induction of IE transcription was dependent upon the presence of a factor that is present in the viral inoculum.

The IE gene transactivation function was mapped to a BamHI fragment of the HSV-1 genome (corresponding to 0.64 to 0.69 map units). This region contains an open reading frame (UL48) that encodes a protein capable of stimulating transcription of the αTK gene in transient transfections of tissue culture cells (Campbell et al., 1984; Pellett et al., 1985). This result revealed that the product of a single viral gene was responsible for IE induction. UL48 represents a leaky late gene (Blair et al., 1987) that encodes a 490 amino acid protein with a predicted molecular weight of 54 kDa (Dalrymple et al., 1985). This protein had previously been identified as

a phosphorylated component of the herpes simplex virion and had been designated VP16 (Spear and Roizman, 1972; Heine et al., 1974) and also Vmw65 (the mobility of the protein as determined by SDS-PAGE corresponds to 65 kDa, Marsden et al.,1976, 1978). Vmw65 is localized to the tegument of the virion which is an amorphous region between the nucleocapsid and the phospholipid bilayer (Roizman and Furlong, 1974). The herpes simplex virus type 2 Vmw65 protein is highly conserved (86 %) relative to the type 1 protein and appears to function in an identical manner (Moss, 1989; Greaves and O'Hare, 1991). The Varicella Zoster Virus (VZV), a member of the herpes virus family, contains a gene, ORF10, encoding a Vmw65 homologue that is 81 amino acids shorter than the HSV-1 protein, lacking the carboxyl terminal acidic domain (Dalrymple et al., 1985). ORF10 does not appear to encode a transcription activator and its true function is not known (McKee et al., 1990). The only other sequence similarity that has been reported is a strong local homology (amino acids 367 to 388) to protein p3 of the bacteriophage ϕ29. This protein is involved in the terminal protein mediated DNA replication process (Haigh et al., 1990). The significance of this homology is not known.

The responsive element that confers the Vmw65 mediated stimulation of transcription was determined to be the enhancer-like element, TAATGARAT (Preston et al., 1984; Gaffney et al., 1985). Significantly, there exists at least

one such element upstream of each of the IE genes. Interestingly, other herpes viruses including VZV, PRV and CMV (human and murine) contain elements that are striking similar to TAATGARAT within their IE promoters (Campbell and Preston, 1987; McKee et al., 1990). Several of these elements are responsive to Vmw65 mediated transactivation (Campbell and Preston, 1987) however no functional homologue to Vmw65 has been identified in any of these viruses. Another element (GCGGAA) located upstream of all IE genes (apRhys et al., 1989) was also determined to play a role in Vmw65 mediated transcription simulation (Triezenberg et al., 1988a,b; reviewed in Thompson and McKnight, 1992). This latter sequence is recognized and bound by a cellular factor which has since been isolated (LaMarco and McKnight, 1989) and cloned (LaMarco et al., 1991). This factor, designated GA Binding Protein (GABP), is composed of two protein subunits one of which (GABPα) contains an Ets-related DNA binding region (Karim et al., 1990) while the other subunit (GABPβ) shows homology to the Drosophila gene Notch. A direct interaction between these proteins and Vmw65 has not been observed (LaMarco and McKnight, 1989) and it has not yet been determined how GABP is involved in Vmw65 mediated transactivation.

UL46 and UL47 are two additional viral genes that have been implicated in Vmw65 mediated transactivation. These ORFs are located just downstream of the UL48 (encoding Vmw65).

Initially, transfection studies had shown UL46 to enhance (2-3 fold) and UL47 to inhibit (2.5-3 fold) Vmw65 mediated transactivation (McKnight *et al.*, 1987a). In the context of the viral genome, however, the deletion of UL46 had no effect and the deletion of UL47 inhibited the induction of αTK relative to wild type virus (Zhang *et al.*, 1991). Neither deletion separately, or together, had any effect on the Vmw65 protein levels or on the ability of the mutated virus to grow in culture (Zhang *et al.*, 1991). UL47 is known to encode a protein that is localized in the virion tegument in quantities comparable to Vmw65 (McLean *et al.*, 1990). It is not known how the UL46 and 47 effect the function of Vmw65 or what significance these effects have on the virus life cycle.

The fact that Vmw65 itself does not have a strong affinity for the TAATGARAT recognition sequence (Marsden *et al.*, 1987) was the first indication that other factors may be required to assist in Vmw65 mediated transactivation. These could potentially be host and/or viral factors. Cellular proteins were shown through mobility shift and footprinting experiments to bind specifically to the recognition sequence (Kristie and Roizman, 1987; Triezenberg *et al.*, 1988b) and these interactions were necessary for the subsequent binding of Vmw65 to the multi-protein-DNA complex (McKnight *et al.*, 1987b; Preston *et al.*, 1988). One of the cellular proteins involved in the protein-DNA complex was identified as an

octamer binding protein (OBP). This protein is similar or identical to a factor that recognizes essential, conserved elements within the promoters of many cellular genes including those encoding histone H2B, U2 snRNA and the immunoglobin light chain as well as an element within the adenovirus origin of replication. An octamer sequence from the immunoglobin kappa light chain promoter was able to confer Vmw65 mediated inducibility on an indicator gene (O'Hare and Goding, 1988). This ubiquitous octamer binding activity has been variously designated NF III (Pruijn et al., 1986), OBP100, (Sturm et al., 1987), TRF (O'Hare and Goding, 1988), OTF-1 (Fletcher et al., 1987) and Oct-1 (Sturm et al., 1988).

Oct-1, as it will be referred to here, is a ubiquitous transcription factor that has been identified in all cell lines tested (Sturm et al., 1987). It is a member of the POU-domain family of DNA binding proteins. The 160 amino acid POU-domain is not only sufficient for the specific recognition and binding of Oct-1 to the octamer sequence but it can also support the formation of the Vmw65 containing multi-protein complex. This domain has been highly conserved between Oct-1 and an otherwise unrelated protein, Oct-2, that is expressed only in lymphoid cells (Clerc et al., 1988; Sturm et al., 1988). Predictably, these two proteins bind indistinguishably to the octamer motif, however Oct-1 is much more efficient at forming a complex with Vmw65 (Kristie et al., 1989). A single

amino acid change in the Oct-2 homeodomain enables Oct-2 to associate with Vmw65 (Lai *et al.*, 1992; Pomerantz *et al.*, 1992). Both proteins contain an N-terminal, glutamine rich region and have been shown to positively regulate transcription. Oct-2 appears to possess an additional intrinsic ability to activate cellular genes that is lacking in Oct-1 (Tanaka and Herr, 1990). It is apparent that Oct-1 supplements its ability to directly activate transcription through interactions with transactivating proteins such as Vmw65. In addition, the phosphorylation of both Oct-1 and Oct-2 is reported to play a role in the regulation of their respective activities (Tanaka and Herr, 1990; Segil *et al.*, 1991).

The octamer motif (consensus ATGCAAAT) is found overlapping the TAATGARAT sequence in several of the IE genes. (TAATGARAT elements containing an octamer overlap have been designated Type II, those without an overlap, Type I, (apRhys *et al.*, 1989)). For example, 7 of the 8 TAATGARAT elements found upstream of the ICP0 and 1 of the 4 ICP4/22 TAATGARATs contain an octamer overlap (apRhys *et al.*, 1989). The full octamer sequence is not required for Oct-1 binding (apRhys *et al.*, 1989) although it may increase the affinity of the protein-DNA interaction (Preston *et al.*, 1988; Dent and Latchman, 1991). Oct-1 binding to TAATGARAT does not stimulate IE gene expression in the absence of Vmw65 (Tanaka *et al.*,

1988). The GARAT sequence does not play a role in Oct-1 binding but is required for the formation of the Vmw65 induced complex (Gerster and Roeder, 1988) and is necessary for the Vmw65 mediated induction of transcription (Latchman, 1991). The above findings suggested that the interaction of Vmw65 with at least one cellular transcription factor (Oct-1) and the TAATGARAT cis-acting element is required to stimulate IE gene expression. There is also the implication that host genes in addition to viral genes are susceptible to induction by Vmw65. This has been demonstrated in the cases of the snRNA gene U3 (Kemp and Latchman, 1988) and U4 (Latchman, 1991).

Mobility shift experiments using purified Oct-1 and Vmw65 revealed that these two proteins alone are incapable of forming the Vmw65 induced complex, suggesting the presence of at least one other factor(s) (Gerster and Roeder, 1988). One of these proteins, or Vmw65 itself, was subsequently implicated in the binding of the GARAT region of the recognition sequence (Goding and O'Hare, 1989). Several factors have been identified which appear to play an essential role in complex formation (Kristie et al., 1989; Katan et al., 1990; Xiao and Capone, 1990; Stern and Herr, 1991). None of these factors have been cloned and therefore their relationship to one another remains unclear. This thesis describes another apparently novel factor involved in Vmw65 induced complex formation.

1.5   Vmw65 and Viral Morphogenesis

Vmw65 is the major tegument polypeptide of the herpes simplex virus and can account for up to 5 % of the total protein content in the herpes virion (Honess and Roizman, 1973). The shear abundance of this protein implies that it may play a role in viral morphogenesis in addition to its role as a transcription factor. The first evidence in support of this hypothesis arose from the characterization of a temperature-sensitive mutant of HSV-2 that mapped to the Vmw65 gene. The mutation had no effect on the ability of this virus to stimulate IE gene expression but did result in the *in vitro* thermolability of the virion (Halliburton and Timbury, 1976; Moss *et al.*, 1979; Moss, 1989). Specific regions of the protein, important for Vmw65's structural function, were identified by testing the ability of mutated genes to rescue the temperature-sensitive defect of this virus. Individual mutations were found that specifically disrupted the ability of Vmw65 to rescue the HSV-2 ts mutant but had no effect on the transactivation function (Ace *et al.*, 1988). These results indicate that Vmw65 is a bi-functional protein that plays an essential structural role that is independent of its capacity as a stimulator of transcription. It is still not clear what role Vmw65 plays in the assembly or in the maintenance of the herpes virion. In the absence of Vmw65, infections with an

HSV-1 Vmw65 deletion mutant (8MA) are non-productive. This mutation has no effect on viral DNA replication or capsid formation and only a slight effect on viral DNA encapsidation. However, a major defect in HSV-1 particle assembly was observed (Weinheimer et al., 1992).

1.6   Vmw65 and its Role in HSV-1 Latency

Latency plays a major role in the HSV-1 life cycle (Fig. 1.5.1) with regard to its persistency of infection and transmission. In contrast to the lytic cycle, in which the viral genome is actively and energetically expressed, no proteins and very few transcripts have been associated with the latent state (reviewed in Roizman and Sears, 1987; Baichwal and Sudgen, 1988). A complete understanding of HSV-1 gene regulation therefore requires an appreciation of the mechanism that determines which of these divergent pathways an infecting virus will take. Vmw65, the initiator of the lytic cycle, may also play an important role in induction of the latent state.

HSV-1 latent infection can be divided into three phases; establishment, maintenance and reactivation. After an initial acute infection at the body's surface, the virus or perhaps just the viral nucleocapsid containing the genomic DNA
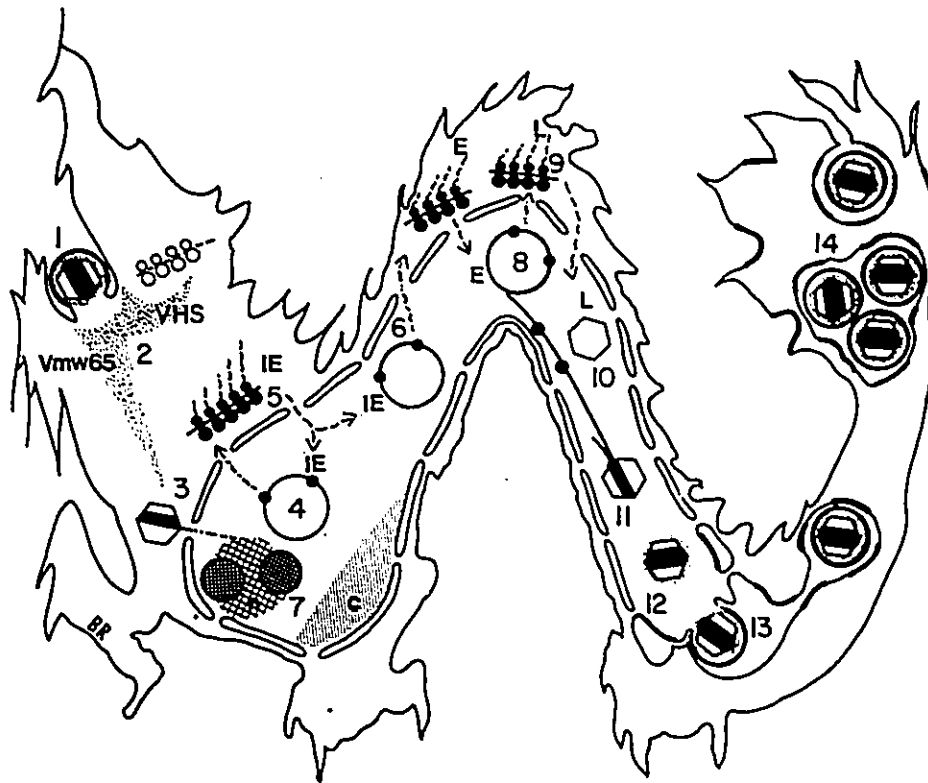
Fig. 1.5.1 Schematic diagram of the HSV-1 infection cycle. (1) The herpes virion enters the sensory neuron and the phospholipid bilayer is incorporated into the plasma membrane. (2) The contents of the virus are released into the cytoplasm; the tegument protein, Vmw65 and the nucleocapsid containing the genomic DNA travels to the nucleus. (3) The nucleocapsid is positioned at a nuclear pore and injects the viral DNA into the nucleus. (4) Immediately upon entry in to the nucleus, the genome circularizes and Vmw65 initiates IE gene expression. (5) The IE proteins regulate their own expression as well as that of the downstream (E and L) genes. (6) The E genes include those which encode factors required for viral DNA replication. (7) The cellular chromatin and nucleoli are broken down. (8) The HSV DNA is replicated as a rolling circle. (9) Many virion structural proteins encoded by the L genes are expressed. (10) Nucleocapsids are assembled in the nucleus (11) and are loaded with a copy of the viral genome. (12) The nucleocapsid matures and (13) exits the nucleus probably through the ER-Golgi membrane systems. (14) The progeny viruses are exported to the surface of the cell where they are released to repeat the cycle. Adapted from Roizman and Sears, 1990.

(Lycke *et al.*, 1984) enters an adjacent sensory nerve ending and travels up the axon to the nucleus of the neuron (Cook *et al.*, 1974). The viral genomic DNA acquires a non-linear, episomal form (Mellerick and Fraser, 1987) and is packaged as chromatin (Javier *et al.*, 1989). In the maintenance stage of latency the viral DNA is detectable but is transcriptionally silent with the exception of the LATs (or Latency Associated Transcripts). LATs have been detected in latently infected sensory neurons of mice (Stevens *et al.*, 1987), rabbits (Rock *et al.*, 1987) and humans (Croen *et al.*, 1987). The major LAT transcript (2.2 kb) contains several short open reading frames that may be joined in-frame in the 1.5 kb LAT splice product. However, there is no evidence for the existence of LAT derived translation products (Wagner *et al.*, 1988a,b). Viruses in which LAT transcription has been compromised still retain the ability to enter, maintain and reactivate from latency (Deshmane and Fraser, 1989). There is some evidence, however, that LAT⁻ mutants are significantly less efficient in the establishment and reactivation stages (Hill *et al.*, 1989; Sawtell and Thompson, 1992).

The latent virus can be reactivated in humans by a wide variety of stimuli including physical or emotional stress, peripheral tissue damage or intake of certain hormones (Roizman and Sears, 1990). The mechanism of reactivation is still poorly understood because of the limitations of the

model systems available. Once activated, the virus replicates and is transported down the axon to a location at or near the point of original entry. The cells of the epithelium are infected in a productive fashion and a lesion forms (Roizman and Sears, 1987).

Much of our knowledge regarding the role of Vmw65 in latent infection has come from work involving the mutant virus *in1814*, constructed and characterized by Ace *et al.*, (1989). *In1814* contains a 12 bp insertion in the Vmw65 coding region which had previously been shown to knock out the ability of the protein to stimulate transcription but does not appear to interfere with viral assembly or the incorporation of Vmw65 into the viral tegument (Ace *et al.*, 1988).

The *in1814* virus does not replicate in the trigeminal ganglia of mice following infection but instead immediately establishes itself in a latent state (Ace *et al.*, 1989). The increased propensity of this mutant virus toward latency may be explained by the observed decrease in the expression of several IE genes (ICP0, 27 and 22). This conclusion is supported by Harris and Preston (1991) who have shown a 5-10 fold reduction in IE transcription is critical in determining whether an infection is lytic or latent. Therefore the presence or absence of the Vmw65 mediated transactivation function may initially play a role in determining the fate of an HSV-1 infection. How the activity of Vmw65 is regulated

during the establishment of latency in a wild type infection
is not clear. It is possible that the virion copies of Vmw65
are dispersed during the long journey up the axon to the
nucleus of the infected neuron.

In terms of reactivation, *in1814* is similar in
frequency and time course to wild-type virus (Ace et al.,
1989) and the pattern of LAT transcription is
indistinguishable from a wild-type infection (Valyi-Nagy et
al., 1991). It has also been demonstrated that Vmw65
expression from an inducible promoter cannot stimulate
reactivation from a latent state (Sears et al., 1991). ICP0
has been shown to be necessary and sufficient for reactivation
in vitro using a human foetal lung cell (HFL) system (Harris
and Preston 1991). It appears that ICP4 is expressed during
reactivation independently of Vmw65 mediated transactivation.
Together these data indicate that although the presence or
absence of transactivationally functional Vmw65 may play a
role in steering an infection toward the lytic or latent
state, respectively, Vmw65 is not required for the
establishment, maintenance or reactivation from latency. One
theory is that the latent state is attained in infected
neurons due to the loss or dispersal of the virion copies of
Vmw65 as the nucleocapsid travels up the axon. The absence of
the transactivator in the infected neural nucleus predisposes
the infection toward latency (Roizman and Sears, 1987).

1.7   The Project

At the time this work began, it was known that the ORF encoding Vmw65 was capable of stimulating viral IE gene transcription through a specific recognition sequence in a transient assay. At the time, there was little or no evidence for the Vmw65 binding to DNA or the involvement of other factors. The existence of highly acidic activation domains within several yeast transcription factors was just being recognized and the generality of this motif within transactivating proteins had yet to be appreciated. Nothing was understood about how such an activation domain could confer an increase in the transcription of a specific gene.

The objective of this research was to determine the mechanism by which Vmw65 mediated the stimulation of gene transcription. Specifically, the goals were:

1) to identify and locate, through mutational analysis, the domains of Vmw65 that are important for activity,

2) to assign specific functions to these domains,

3) to identify and characterize viral and cellular factors that are involved in the Vmw65 transactivation function,

4) to begin to investigate the role played by Vmw65 in the course of a herpes simplex virus lytic infection.

It was hoped that the study of this transactivator would increase the understanding of eukaryotic gene regulation in

general as well as the specific role of Vmw65 in the herpes simplex infection cycle. The major advantage of this particular system was that the effector protein, Vmw65, and the enhancer sequence target, TAATGARAT, had been clearly identified. The intensive and concurrent investigation of Vmw65 mediated transactivation by many different laboratories over the past few years have established it as a prototypical system for the study of transcription regulation. The identification and examination of the Vmw65 acidic activation domain has facilitated the study of the mechanism by which this common activator domain functions (Laybourn and Kadonaga, 1991; Lin and Green, 1991; White et al., 1992). The close interaction of Vmw65 with the cellular transcription machinery provides an avenue by which the study of this viral protein can be expanded into the examination of the basis of RNA polymerase II transcription.

This thesis is divided into three sections. The first describes the methods and discusses the results and implications of the structure-function analysis of Vmw65. The second section is concentrated upon the identification and characterization of factors that are involved or influence the assembly of Vmw65 into the multi-protein-DNA complex required for transactivation (with an emphasis on one factor in particular that was first identified in this work). Finally, the third section describes the construction and

characterization of a Vmw65-producing cell line that was utilized to examine the effect the presence of this protein has on the course of infections with wild type and mutant virus as well as HSV-1 genomic DNA.

Much of what is known of Vmw65 and its role as a transcription activator has come from the large number of concurrent studies from other laboratories. This information has been appropriately integrated with the results of this study in an attempt to give a broad view of the field as it exists today.

MATERIALS AND METHODS

2.1.1   Chemicals

The following is a list of critical chemicals and reagents and the companies from which they were purchased.

ampicillin	Sigma Chemical Company
bacto-agar	Difco Laboratories
BioRad protein assay dye reagent	BioRad Laboratories Ltd.
5-bromo-4-choro-3-indolyl β-D-galactoside (X-gal)	Gibco/BRL
5-bromo-4-choro-3-indolyl phosphate (BCIP)	Promega Corporation
Cromassie Brilliant Blue	BRL
deoxyribonucleotides	Pharmacia Inc.
dimethyl sulfoxide	BDH Chemicals
gentamycin (G418)	Gibco/BRL
isopropyl β-D-thiogalactoside (IPTG)	Gibco/BRL
molecular weight standards	
-1kb ladder	Gibco/BRL
-SDS-PAGE standards	Sigma Chemical Company
NA45 nitrocellulose paper	Schleicher & Schuell
nitro blue tetrazolium (NBT)	Promega Company
Nonidet P-40 (NP-40)	Sigma Chemical Company
NuSieve agarose	FMC Bioproducts
Sarkosyl	BRL
Sephadex G-10,15,25,50	Pharmacia
Silica gel 150A plates	Whatman
Triton X-100	Sigma Chemical Company
Tween 20	Sigma Chemical Company

2.1.2 Radiochemicals

Chloramphenicol, [dichloroacetyl-1,2-^{14}C]- (58-60mCi/mmol) was purchased from New England Nuclear. [α-^{32}P]-dATP (3000 Ci/mmol) was obtained from ICN Radiochemicals though the laboratory of Dr.C.Harley, Department of Biochemistry, McMaster University. [γ-^{32}P]-ATP (3000 Ci/mmol) was obtained from New England Nuclear through the laboratory of Dr.P.Branton, Department of Pathology, McMaster University.

2.1.3 Enzymes

Restriction endonucleases were purchased from BRL, Boehringer Mannheim, New England Biolabs or Pharmacia. Klenow, T4 DNA ligase, and polynucleotide kinase were purchased from BRL or Pharmacia. Proteinase K, DNAse I and RNAse A were purchased from Pharmacia. Trypsin was purchased from Gibco. All exoglycosidases and aryl sulfatase were obtained from Beohringer Mannheim or Sigma. Phospholipase A$_2$ was provided by Dr.G.Gerber, Department of Biochemistry, McMaster University. All enzymes were used according to the manufacturers' instructions.

2.1.4   Antisera

LP-1, a monoclonal antibody targeted to the N-terminal domain of Vmw65 was provided by Dr.T.Minson, Department of Pathology, University of Cambridge. Affinity purified Goat anti-Mouse IgG Alkaline Phosphatase conjugated antibody was purchased from BioRad.

2.1.5   Cell lines and viral strains

BSC40, Vero and HeLa S3 cells were obtained from the American Type-Culture Collection, Rockville, Maryland. HSV-1 strain 17 (Syn⁺) was provided by Dr.D.Johnson, Department of Pathology, McMaster University. and HSV-1 in1814 was obtained from Dr.C.Preston, MRC Virology Unit, Glasgow, Scotland. Fm3a cells were obtained from Dr. J.A. Hassel, Institute of Molecular Biology, McMaster University.

2.1.6   Cloning vectors and bacterial strains

Plasmid pMC1 and pGX158 were obtained from Dr. C. Preston, Medical Research Council Virology Unit, Institute of Virology and contains the entire coding region of Vmw65 from strain 17 (Campbell et al., 1984; Dalrymple et al., 1985). p175cat was constructed from pRSVcat (Gorman et al., 1982) by

Dr. J. Capone. p38Kcat (O'Hare and Hayward, 1984) was obtained from Dr. G.Hayward, Department of Pharmacology and Molecular Science, John Hopkins School of Medicine. pZIPneoSV(X)1 (Cepko et al., 1984) and ptsA58 (Sedivy et al., 1982) were obtained from Dr. R.C. Mulligan, Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology. pBSOCT-1 was supplied by Dr. W. Herr, Cold Spring Harbour. pSPUTK was obtained from Dr. D. Andrews, Department of Biochemistry, McMaster University. pRIT2T (Nilsson et al., 1985) and pGEX2T (Smith and Johson, 1988) were purchased from Pharmacia.

All cloning was performed in E.coli strains HB101 (supE44hsd520($r_b^-m_b^-$)recA13ara-14proA2lacY1galK2rpsL20xyl-5mH-1) or DH5α (supE44ΔlacU169Φ80lacZΔM15)hsdR17recAlendA1gyrA96 thi-1relA1) which were obtained from Gibco/BRL. The pRIT2T vectors were transformed into the strain N4130 (F'su'his'ilv' galKΔ8Δ(chlD-pgl)λΔBamN+cI_{857}ΔH1) purchased from Pharmacia.

2.1.7   Oligonucleotides

The oligonucleotides listed in Table 2.1.1 were obtained from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University with the exception of 3 (BamHI linker) which was purchased from Pharmacia and AB 655/656 which was provided by Dr. J.A. Hassel, Institute of Molecular Biology, McMaster University.

OLIGO	SEQUENCE	PURPOSE
3	5'-CCCGGATCCGGG-3'	BamHI linker for insertion mutagenesis
AB164	5'-CTAGCTAGCTAG-3'	Stop codon linker for mutagenesis
$(dT)_{18}$	5'-TTTTTTTTTTTTTTTTTT-3'	Northern blot control probe
AB580	5'-GATCCCGTGCATGCTAATGATATTCTTT-3'	WT mobility shift probe from ICP0 promoter (two strands)
AB581	5'-CTAGAAAGAATATCATTAGCATGCACGG-3'	
AB582	5'-GATCCCGTGCCTGATCATGATATTCTTT-3'	OCT⁻ mobility shift probe (two strands)
AB583	5'-CTAGAAAGAATATCATGATCAGGCACGG-3'	
AB584	5'-GATCCCGTGCATGCTAATTACCGTCTTT-3'	GARAT⁻ mobility shift probe (two strands)
AB585	5'-CTAGAAAGACGGTAATTAGCATGCACGG-3'	
AB721	5'-GATCTTCGGCGGTAATGAGATACGAG-3'	WT mobility shift probe from ICP4 promoter (two strands)
AB722	5'-GATCCTCGTATCTCATTACCGCCGAA-3'	
AB655	5'-GATCTGACTAACTGAGCACAGG-3'	AP1 mobility shift probe (two strands)
AB656	5'-GATCCCTGTGCTCAGTTAGTCA-3'	

Fig. 2.1.1   List of Oligonucleotides

2.2   Cloning

2.2.1   Preparing transformation competent *E. coli*

100 ml cultures of the appropriate bacteria (ie HB101, DH5α) were grown to an OD of 0.4-0.5. These were pelleted and resuspended in 20 ml of RF-1 (contains 100 mM RbCl, 50 mM MnCl$_2$, 30 mM KOAc, 10 mM CaCl$_2$ and 15 % glycerol adjusted to pH 5.8 with 0.2 M HAc). After a 1 hour incubation on ice the bacteria were again pelleted and resuspended in 8 ml RF-2 (contains 10 mM MOPS, 10 mM RbCl, 75 mM CaCl$_2$ and 15 % glycerol adjusted to pH 6.8 with NaOH). The cells were then divided into 200 μl aliquots, flash frozen in liquid nitrogen and stored at -80°C.

2.2.2   Transformation of *E. coli*

Approximately 0.1 μg of plasmid DNA was added to 200 μl of competent *E. coli* and incubated on ice. After 40 minutes the cells were heat shocked at 42°C for 2 minutes. 2 ml of 2YT was added and the cells were incubated at 37°C with shaking for 1-1.5 hours. At this time 50-200 μl aliquots of the culture were spread onto plates containing 2YT, 1.5 % bactoagar and 50 μg/ml ampicillin (if blue/white screening, each plate would also contain 50 μl of 2 % X-gal in

dimethylformamide and 10 μl of 100 mM IPTG). The plates were incubated overnight at 37°C.

2.2.3   Preparation of plasmid DNA

Plasmid DNA was prepared on a small scale by the boiling method as described in Sambrook *et al.* (1989) or in large quantities using a alkaline lysis technique (Sambrook *et al.*, 1989). Minipreparations of DNA were isolated from 4 ml of bacterial culture grown overnight in 2YT. 1.5 ml of cells were pelleted by centrifugation in an eppendorf tube. The pellet was resuspended in 350 μl of mini-prep lysis buffer containing 8% sucrose, 50mM EDTA, 10mM Tris-HCl pH 8.0 and 0.5 % Triton X-100. To this 30 μl of freshly prepared 10 mg/ml lysozyme was added. The tube was then vortexed vigorously and boiled for 60 seconds. Cell debris was pelleted by centrifuging for 10 minutes in a microfuge and then removed with a toothpick. 200 μl of 7.5 M NH$_4$Ac and 700 μl of isopropanol were added to the supernatant and the DNA was allowed to precipitate for 30 minutes at -20°C. The plasmid DNA was then pelleted in a microfuge and resuspended in 50 μl of TE.

Large scale plasmid preparation involved growing a 500 ml bacterial culture overnight in the presence of 50 μg/ml ampicillin. The bacteria were then pelleted and resuspended in 36 ml of GTE containing 50 mM glucose, 25 mM Tris pH 8.0 and

10 mM EDTA. To this was added 4 ml of 5 mg/ml lysozyme (fresh) and the mixture was allowed to sit at room temperature for 10 minutes. The bacteria were lysed with the addition of 80 ml of 0.2 M NaOH containing 1 % SDS and incubated on ice for 5 minutes. 40 ml of 5 M KOAc was added before another 20 minute incubation on ice. The mixture was then centrifuged at 9 000 rpm in a GSA rotor for 15 minutes. The supernatant was decanted though 4 layers of cheesecloth and then 400 ml of isopropanol was added. The precipitated nucleic acids were pelleted by centrifugation and resuspended in 7.5 ml of TE. To this 8.8 g of CsCl and 1.0 ml 10 mg/ml ethidium bromide were added before incubating on ice for 15 minutes. The supernatant from a 7 000 g, 30 minute spin was then loaded into : Beckman Quick-Seal ultracentrifugation tubes and centrifuged in a VTi65 rotor at 15°C and 55 000 rpm for 20 hours. The plasmid DNA was recovered from the tube by side puncture with a 3 ml syringe and an 18 gauge needle. The ethidium bromide was removed by repeated extractions with H_2O/CsCl saturated butanol. The solution was dialysed against TE and then ethanol precipitated.

2.3   Mutagenesis

2.3.1   Construction of Linker Insertion and Deletion Mutants

pMC1 was digested with *Hae*III, *Rsa*I or *Mlu*I under conditions predetermined to optimize the production of linearized (single cut) plasmid. This was accomplished by adding 80, 200 and 150 μg/ml, respectively, of ethidium bromide to a normal one hour digestion (Shortle and Botstein, 1983). Full length molecules were isolated from an agarose gel using the Gene Clean *kit* (Bio/Can Scientific) based on the methods of Vogelstein and Gillespie (1979). A *Bam*HI linker 5'pCCCGGATCCGGG, was ligated to the plasmid using T4 DNA ligase. Multiple linkers were removed by extensive *Bam*HI digestion and the plasmid was re-ligated and used to transform *E.coli* HB101. Insert sites were mapped by restriction analysis on agarose and polyacrylamide gels. pN299 was constructed by inserting the *Bam*HI linker into the *Nru*I site at nucleotide position 1346 of pMC1.

In-frame deletion mutants were produced by ligating the 5'end of one linker insert to the 3'end of an appropriately chosen linker insert located downstream of the first. One fortuitously obtained in-frame deletion was isolated during the screening of the linker insertion mutants. pΔSal was obtained from pMC1 by deletion of the 1217-bp

fragment spanning the two *SalI* sites in the Vmw65 reading frame.

2.3.2   Construction of Premature Chain Termination Mutants

Nonsense mutants were obtained by cutting selected linker insertion mutants with *BamHI* and then producing blunt ends with Klenow and the dNTPs. The synthetic oligonucleotide AB164 (5'pCTAGCTAGCTAG), containing stop codons in all three reading frames was then ligated into the plasmid. The diagnostic NheI site within the linker was utilized to screen for its presence. pAm186 is a pMC1 derived mutant that contains an amber stop codon in place of serine 186 This construct was produced by John Capone using cassette mutagenesis. Sequences between the *KpnI* site and the *PvuII* site (nt positions 1006 and 1017, respectively) were replaced with the synthetic oligo 5'pCTGCGCGCCTAGGTCCGGCAG and its complement, 5'pCTGCCGGACCTAGGCGCGCAGGTAC and the construct was confirmed by dideoxy sequence analysis.

2.4   Expression Systems

2.4.1   The protein A fusion system: cloning

The 1217 bp *Sal*I fragment contained with the coding region of Vmw65 from the plasmid pMC1 was ligated into the unique SalI site of pRIT2T (Nilsson *et al.*, 1985) so that amino acids 5-411 of Vmw65 would be in-frame with the N-terminal, IgG binding domains of protein A. This plasmid was designated pRITsal. Select Vmw65 linker insertion, deletion and nonsense mutants were transferred from the pMC1 plasmid to pRIT2T in a similar way. In all cases the correct orientation of the insert was determined by diagnostic restriction digests. pRIT65 containing the entire coding sequence of Vmw65 with the exception of the first four amino acids was constructed by ligating the 1417 bp KpnI fragment from pMC1 into the unique *Kpn*I site in pRITsal.

pRIT-POU (constructed by P. Xiao) contains the POU/homeodomain of Oct-1 (amino acids 271-441) fused in-frame behind the protein A IgG binding domain. It was constructed by ligating a *Bam*HI linker to the *Hinc*II fragment of pBSOct-1 (Sturm *et al.*, 1988) and then ligating an adapter containing 2 stop codons (GTTAACAAGCTTAG) to the *Pfl*mI site just downstream of the homeodomain. Following digestion with *Bam*HI, the fragment was ligated into the *Bam*HI-*Eco*RV sites of pRIT2T.

2.4.2   Purification of protein A fusion proteins

N4830-1 cells (Pharmacia) transformed with the appropriate construct (described above) were grown at 30°C to an OD of 0.6. At this time protein production was induced by adding an equal volume of 2YT which had been heated to 55°C and the cells were incubated at 30°C for an additional 2 hours. The cells were pelleted, washed and then resuspended in a buffer containing 13 % sucrose, 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 10 mM EDTA, 0.1 % NP40, 1 mM PMSF and 0.25 mg/ml lysozyme. After a 30 minute incubation on ice the lysed cells were centrifuged at 40 000 for 1.5 hours in a Beckman 50.2 rotor. The supernatant was applied to a 3 ml IgG Sepharose 6FF (Pharmacia) column using a Masterflex pump (Cole-Parmer Instrument Company). The column was washed with 50-100 ml of TST (50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.05 % Tween 20) and then with 10 ml of 5 mM NH_4Ac pH 5.0. The fusion protein was then eluted with 10 ml of 0.5 mM HAc, pH 3.4. 1 ml fractions were collected, tested for protein content using the BioRad assay and the protein containing fractions were dialysed against Buffer E (50 mM Tris-HCl pH 7.6, 50 mM NaCl, 0.5 mM DTT, 1 mM PMSF and 10 % glycerol) or alternatively exchanged into Buffer E using PD 10 Columns (Pharmacia).

2.4.3   The Glutathione S-Transferase (GST) fusion system: cloning

pGEX-SPUTK was constructed by Dr. Capone by the insertion of the *Bgl*II-*Eco*RI polylinker from pSPUTK into the *Bgl*II-*Eco*RI sites of pGEX2T (Smith and Johnson, 1988). The 1762 bp *Nco*I-*Eco*RI fragment from pSPUTK-65 was inserted into the *Nco*I-*Eco*RI sites of pGEX-SPUTK so that the glutathione-binding domain of GST would be expressed as an in-frame fusion protein with full length Vmw65. The pGEX plasmids contain an IPTG inducible *tac* promoter.

2.4.4   Purification of GST fusion proteins

Cells transformed with the appropriate pGEX plasmid were grown to an OD of 0.6 and then protein production was induced by the addition of 0.2 mM IPTG. After incubating an additional 2 hours the cells were pelleted, washed and resuspended in PBS containing 0.1 % Triton X-100. The lysed cells were then centrifuged at 10 000 rpm for 20 minutes. The supernatant was applied to a column packed with 3 ml Glutathione Sepharose 4B (Pharmacia) and the column was washed with 50-100 ml of PBS containing 0.05 % NP40. The GST fusion protein was eluted with 10 ml of 50 mM Tris-HCl pH 8.0 containing 5 mM Glutathione (Sigma). 1 ml fractions were collected and the protein concentration of each was determined

using the BioRad assay. Protein containg fractions were dialysed against Buffer E.

2.5 Cell Culture

2.5.1 Maintenance

BSC40 and Vero cell lines were maintained in Dulbecco modified essential medium supplemented with 10 % calf serum, 1 % penicillin/streptomycin and 2 % glutamine on 10 cm dishes. BSV65 cells were maintained under similar conditions in the presence of 600 μg/ml G418. Upon reaching confluence cells were treated with trypsin and diluted 1:10 into fresh medium. HeLa cells were maintained in Joklik medium supplemented with 5 % fetal calf serum, 1 % penicillin/streptomycin, 2 % glutamine, 1X vitamins and 1 % non-essential amino acids. Cell density was maintained between 2.0×10^5 and 5.0×10^5 cells/ml by subculturing every 2-3 days as required.

2.5.2 Transfections

Transfections were carried out essentially as described by Graham and Van der Eb (1973). Plates of cells were transfected when they were approximately 80 % confluent.

The plasmid DNA along with salmon testes DNA (to make the total DNA content 20 μg) were mixed together in an eppendorf tube. To this 62 μl of 2 M $CaCl_2$ and H_2O up to 0.25 ml was added. This mixture was then added drop-wise to 0.25 ml of 2X HBS (over 30 seconds) while vortexing the HBS (HBS contains 0.28 M NaCl, 50 mM HEPES, 1.5 mM sodium phosphate adjusted to pH 7.12 with 0.5 M NaOH). The mixture was then allowed to sit for 30 minutes at room temperature. The DNA precipitate was added drop-wise to the 5 ml of fresh medium covering the cells. The plates were aspirated after 6 hours and the cells were shocked with serum free medium containing 10 % dimethyl sulfoxide. The cells were then harvested after 48 hours.

Transfections with HSV-1 genomic DNA differed in that the HSV-1 (50 μg) was added to 250 μl of 2XHBS and H_2O up to 475 ml. To this solution 25 μl of 2 M $CaCl_2$ was added. After a 20 minute incubation at room temperature the precipitated DNA was added drop-wise to the cells. Viral plaques were observed 48 hours later.

2.5.3   Isolation of cell lines expressing Vmw65

BSC40 cells at 80 % confluence were co-transfected with 10 μg pZIP65, 0.5 μg of pSV2neo and 9.5 μg of salmon testes DNA using the calcium phosphate method (Graham and van der Eb, 1973). After 6 hours at 37°C the cells were shocked

using serum-free medium containing 10 % dimethyl sulfoxide. The cells were trypsinized and split 1:4 after 24 hours. The next day the cells were washed and incubated in Dulbecco medium containing 800 μg/ml G418. Individual G418 resistant colonies were isolated after 14 days of selection (with medium changes every 3 days). These were picked (trypsinized), expanded and tested for Vmw65 activity using transient transfections with p175cat or p38Kcat. The cell line BSV65 was selected for its ability to specifically and significantly stimulate transcription through the IE promoter.

2.5.4   Isolation of DNA from tissue culture cells

Cells were trypsinized, pelleted and resuspended in 1 ml STE (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA) per 10 cm dish of cells. The solution was made to 0.5 % SDS, 500 μg/ml proteinase K and 100 μg/ml RNAse A (final concentrations) and heated to 65°C for 2 hours. The DNA was phenol-chloroform extracted, ethanol precipitated and resuspended in TE.

2.5.5   Isolation of RNA from tissue culture cells

The harvested cell pellet was lysed in 2 ml ice cold GIT/10^8 cells (GIT is composed of 4 mM Guanidinium

Isothiocyanate, 25 mM sodium citrate, 0.1 M β-Mercaptoethanol) with two 20 second sonications. The cell extract was layered onto a 3 ml 5.7 M CsCl cushion and the tubes were filled to the top with GIT. After 12 hour at 32000 rpm in the SW40.1 rotor the solution was carefully aspirated off. The pellet was resuspended in 0.5 % SDS, heated to 55°C for 5 minutes, cooled on ice and spun in a microfuge for 5 minutes. The supernatant was ethanol precipitated and resuspended in H_2O.

2.5.6   Viral infections and plaque assays

Confluent 10 ml plates of Vero cells were washed 3 times with PBS. The appropriate aliquot of virus was added to the cells in 3 ml of medium. After a 2 hour incubation at 37°C, to allow for viral adsorption, 10 ml of medium was added back to the cells and the infection was allowed to proceed for the desired time.

Plaque assays typically required a six well trays of Vero cells (confluent). Virus stocks were diluted 10^2 to 10^9 times (increments of 10 fold) in 1 ml of DME without serum. The presence of serum inhibits viral adsorption onto the cells. Medium was removed from the cells and 0.2 ml of each virus dilution was added (a mock infection contained DME without serum with no virus). Cells were incubated at 37° C and were rocked every 15 minutes. After 2 hours, 3 ml of DME

complete containing 0.05 % HGG (human gamma globulin, to prevent virus spreading) was added and the infection was allowed to proceed 2 to 3 days. Plaques were counted to determine the virus titre, expressed as PFU/ml.

2.5.7 Construnction and isolation of a virus deleted of the Vmw65 ORF

The plasmid pVP16ßgal was constructed (by Grant Moore) by replacing the 1.2 kb *HincII* fragment containing the Vmw65 coding region of pH34 with the 3.3 kb *SmaI-ScaI* fragment from pMC1871 containing a ß-*gal* gene (Casadaban et *al.*, 1983) so that ß-*gal* was expressed from the Vmw65 promoter. 20 µg of pVP16ßgal and 20 µg of HSV-1 strain 17 genomic DNA were cotransfected into confluent plates of BSV65 cells by the calcium phosphate method. Two days post-transfection cells were overlayed with 1 % agarose and 30 µg/ml X-Gal in 1 X MEM medium. Blue plaques were picked and suspended in 1 ml of MEM medium for 30 minutes at 4°C to allow the virus paricles to diffuse out of the gel. Plugs were then freeze-thawed two times and the sonicated three times for 10 second intervals with a probe sonicator. The virus was then titred on BSV65 cells or stored at -80°C.

2.5.8   Isolation of DNA from virus particles

Viral DNA was isolated by the Hirt extraction method (Hirt, 1967). Typically, confluent, 10 cm plates of Vero cells were infected with 10^6 PFU virus. At the time of total CPE (cytopathological effect), cells were harvested in 15 ml PBS and pelletted by centrifugation (300 g for 10 minutes). The pellet was resuspended in 0.5 ml of 10 mM Tris, pH 7.6 and 10 mM EDTA and transferred to an eppendorf tube. 30 μl of 10 % SDS was added and cells were gently mixed until they lysed. 132 μl 5 M NaCl was then added, cells were gently mixed and incubated at 0°C overnight. The next day, the tubes were microfuged for 5 minutes at 4°C. The supernatant was removed and phenol extracted 3 times, then ether extracted 2 times. The aqueous volume was adjusted to 0.5 ml with water and 1.25 ml 95 % EtOH was added. DNA was precipitated at -20 °C overnight. Pelletted DNA was resuspended in 50-100 μl TE containing 10 μg/ml RNAse.

2.6   Chloramphenicol Acetyltransferase Assay

Cells were harvested by scraping with a rubber policeman into 1 ml of TEN buffer (40 mM Tris-HCl pH 7.4, 1 mM EDTA and 0.15 M NaCl). The cells were pelleted and resuspended

in 100 μl CAT assay buffer (containing 0.25 M Tris-HCl pH 7.8, 0.5 % NP40 and 1 mM PMSF). Cells were lysed by vortexing and sonication and the supernatant was collected after a 5 minute spin. All extracts were normalized for protein content.

Chloramphenicol acetyl transferase activity was determined in 10-50 μl of the supernatant to which the following were added; 1 μl [^{14}C]-chloramphenicol, 70 μl 1 M Tris-HCl pH 7.8, 25 μl 5 mM acetyl CoA and H_2O to a final volume of 150 μl. This solution was incubated for 1 hour at 37°C after which the reaction was terminated by the addition of 1 ml of ethyl acetate. The ethyl acetate phase containing the chloramphenicol was separated by centrifugation and dried down in a Speed Vac. The pellet was resuspended in 25 μl ethyl acetate, spotted onto prescored silica gel plates (Whatman) and run in a solvent containing a 95:5 ratio of chloroform to methanol. Plates were dried and exposed to film overnight. Radioactive spots were recovered and quantitated by liquid scintillation counting.

2.7 Preparation of Extracts From Tissue Culture

2.7.1 Preparation of nuclear extracts from tissue culture cells

Nuclear extracts were prepared from monolayers of BSC40 and BSV65 cells and form suspension cultures of HeLa and Fm3a cells as described by Dignam et al. (1981). Post-nuclear (S100) extracts were prepared as described in the same source. The HeLa extracts were prepared from 3 L of culture at a density of $4-6 \times 10^5$ cells/ml and would yield approximately 20 mg of protein. The S100 extract would yield an additional 100-150 mg of protein as determined by the BioRad assay.

Typically, cells were washed in PBS and then resuspended in 5 PCVs (pellet containing volumes) of Buffer A (containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT) and allowed to swell for 5 minutes on ice. The cells were then re-pelleted and suspended in 2 PCVs of Buffer A. Lyses was accomplished with at least 10 strokes using a dounce pestle B. After centrifugation for 10 minutes at 500 g the supernatant was removed and used to prepare an S100 extract (see below). The pellet was then again centrifuged for 20 minutes at 25 000 g and resuspended in 3ml of Buffer $C/10^9$ cells (Buffer C contains 20mM HEPES, pH 7.9, 25 % glycerol, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1.5 mM $MgCl_2$ and 0.5 mM DTT). The cell extract was stirred gently for 30 minutes at

4°C and then centrifuged for 30 minutes at 25 000 g. The supernatant was dialysed for 12 hours against 50 volumes of Buffer D (20 mM HEPES, pH 7.9, 20 % glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT) and then again centrifuged for 20 minutes at 25 000 g.

2.7.2   Preparation of post-nuclear extracts

The nuclear extract was aliquoted in small volumes and stored at -80°C. To prepare an S100 extract 0.1 volumes of Buffer B (0.3 M HEPES, pH 7.9, 1.4 M KCl and 30 mM MgCl$_2$) were added to the post-nuclear extract (prepared as described above). The extract was then centrifuged at 100 000 g for 60 minutes and dialysed against 20 volumes of Buffer D. S100 extracts were aliquoted and stored at -80°C.

2.7.3   The fractionation of S100 extracts

HeLa cell S100 extract was fractionated by sequential ultrafiltration though a series of Centricon filters (Amicon) having decreasing molecular weight cut-offs. 4 ml S100 extract (40 mg/ml) was centrifuged in a Centricon 30 (MW cut-off 30 kDa) for 4 hr at 4000 g. 3.5 ml of the filtrate was centrifuged in a Centricon 10 (MW cut-off 10 kDa) for 2 hr at 4000 g. 3 ml of the C10 filtrate was loaded into a Centricon

3 filter (MW cut-off 3 kDa) and centrifuged for 6 hr at 7500 g. The final volume of C3 filtrate was 2.5 ml. The retentates from each spin were adjusted with buffer A to their original volumes and were tested, along with the filtrates, for their ability to stimulate Vmw65-dependent complex formation.

2 ml of the active C3 filtrate was subsequently fractionated using DEAE Sephacel ion exchange chromatography. The activity was step eluted with 1.25-1.5 M KCl in a 2 ml volume and then desalted on a Sephadex G-15 gel filtration column from which it was excluded. Finally, a Bligh Dyer (MeOH/CHCl$_3$) extraction was performed on the active fraction (Bligh and Dyer, 1989); 1 ml of the fraction was mixed with 3.75 ml of a 1:2 MeOH/CHCl$_3$ solution, after which 1.25 ml CHCl$_3$ and 1.25 ml H$_2$O were sequentially added. Aqueous and organic phases were separated and then concentrated under vacuum (to 1 ml). The enhancing activity (SF) was found to partition into the aqueous and not the organic phase.

The SF fraction was further separated by Thin Layer Chromatography (TLC). Aliquots of 10 to 20 μl of the most-pure fraction containing enhancing activity were spotted on Whatman 5K LK5D silica plates and allowed to dry. The fraction was separated with four ascents of a solvent consisting of acetonitrile-water (85:15, v/v). Carbohydrates were visualized by spraying the dried plate with sulphuric acid-methanol (1:3, v/v) followed by heating for 10 minutes at 110-120°C.

Carbohydrates appear as black or brown spots (Robyt and White, 1987). Amines were visualized by spraying the plate with a solution of 300 mg of ninhydrin, 3 ml of glacial acetic acids and 100 ml of butyl alcohol heated 10 minutes at 110°C (Robyt and White, 1987).

2.7.4 Preparation of fractions containing VCAF-1 activity

The VCAF-1 fractions utilized were prepared by Peter Xiao and Bilyana Popova from rat liver nuclear extracts. Typically, 5 mg of rat liver nuclear extract (prepared as described by Hattori et al., 1990) was separated on a BioRad Econo Pack anion exchange Q column. VCAF-1 activity was eluted with a linear gradient of KCl from 150-250 mM at an elution velocity of 0.5 ml/min. The VCAF-1 fractions typically contained a protein concentration of 75 μg/ml. The fractions were subsequently dialysed against 25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10 % glycerol and 1 mM DTT.

2.7.5 Preparation of viral protein extracts

Ten confluent 15 cm plates of Verc cells were infected with HSV-1 strain 17 at an moi of 20. The cells were incubated for 24 hours at 37°C and then harvested by scraping with a rubber policeman. The pelleted cells were resuspended in 5 ml

of supernatant, freeze-thawed three times and then sonicated for 20 seconds. Cell debris was removed by centrifugation (500 g for 10 minutes) and the virus was pelleted in a 2 hour spin at 30000 g (16000 rpm in Type 19 rotor). The pellets were resuspended in PBS containing 0.5 % calf serum and spun though a 2 ml 10 % PBS sucrose cushion for 1 hour at 40 000 rpm in an Beckman SW41 rotor. The pellet was resuspended in 1.5 ml of a solution containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl and 1 mM EDTA and NP40 was added to a final concentration of 0.03 % (v/v). This mixture was incubated on ice for 1 hour and then spun at 30 000 rpm for 1 hour in a Beckman SW50.1 rotor. The supernatant was removed, glycerol was added to 10 % final concentration and the extract was stored in 200 μl aliquots at $-80°C$.

2.8   Analysis of Nucleic Acids

2.8.1   Preparing and labelling DNA probes

2.8.1.1 Labelling Southern probes

The 1.2 kilobase *Sal*I fragment contained in the coding region of Vmw65 from pMC1 was labelled using random priming (Feinberg and Vogelstein, 1983, 1984). 50 ng of DNA was

boiled, quick cooled and then incubated with 25 μCi [α-^{32}P]dATP, 1 unit of Klenow and 10μl of 5X OLB buffer for 5 hours at room temperature . OLB buffer is composed of Solutions A, B and C mixed in a ratio of 0.2:0.5:0.3 (v,v,v). Solution A consists of 1.25 M Tris-HCl, pH 8.0, 0.125 M MgCl$_2$, 0.25 M β-mercaptoethanol and 0.5 mM each of dCTP, dGTP and dTTP. Solution B contains 2 M HEPES, pH 6.6 and Solution C contains hexadeoxyribonucleotides (90 OD/ml in TE) purchased from Pharmacia. The reaction was stopped with 200 μl of stop solution (20 mM NaCl, 20 mM Tris-HCl, 2mM EDTA, 0.2 % SDS and 1 μM dATP). The labelled DNA was purified using Spun Columns (BioRad). The DNA fragments produced by the *Bam*HI digestion of pVp16βgal were labelled in a similar manner.

2.8.1.2 Labelling northern probes

The 1.8 kilobase *Bam*HI fragment contained within the coding region of the HSV-1 Vmw175 gene was isolated from pRHP6 and labelled by random primer extension (Feinberg and Vogelstein, 1983,1984) as described above. 10 pmoles oligo (dT) was 5'-end-labelled by incubation with 10 μCi [γ-^{32}P]-dATP and 5 units T4 polynucleotide kinase in 50mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$ and 5 mM DTT for 1 hour at 37°C in a 50 μl reaction volume. The probe was Spun Column purified.

2.8.1.3 Labelling mobility shift probes

Synthetic oligonucleotides AB580/1, AB582/3, AB584/5 and AB720/1 were annealed by heating to 95°C and then slow cooling over 5 hours to room temperature in the presence of 50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$. In the labelling reaction 15 pmoles of annealed oligo were incubated with 100 μCi $[\alpha-^{32}P]$-dATP, 20nM of each dCTP, dGTP and dTTP, and 5 units of Klenow for 2 hours at room temperature. The reaction was chased with 20 nM of cold dATP for 10 minutes. The probe was resolved on a 4 % Nuseive Agarose gel and electrophoretically run onto S+SN45 DEAE ion exchange nitrocellulose paper. The probe was eluted from the paper by incubating in 1.25 M NaCl at 68°C for 30 minutes. The eluted probe was then ethanol precipitated and resuspended in 100 μl ddH_2O.

2.8.1.4 The DNAse I footprint probes

Plasmid pSPUTK-TAAT or pSPUTK-Oc⁻, containing the mobility shift oligos AB580/1 and AB582/3 respectively (cloned into the *BamHI/XbaI* sites of pSPUTK), was digested with *EcoRI*. The 5'-overhang was filled in using Klenow, $[\alpha-^{32}P]$-ATP and dTTP. After the Klenow was heat inactivated a 129 bp DNA fragment was liberated from the plasmid by digesting with *HinDIII*. The probe was gel purified onto nitrocellulose paper

as previously described. Probes in which the opposite strand of DNA was labelled were produced by first cleaving the DNA with *Nco*I, end labelling with Klenow and then cutting with *Hpa*I. This liberated a 134 bp fragment which was isolated and purified.

2.8.2   DNA purification

DNA fragments were separated and analyzed by agarose gel electrophoresis as described by Sambrook *et al.*, (1989). Lambda DNA cleaved with *Hin*DIII and *Eco*RI or a 1 kb ladder (BRL) were utilized as size markers. DNA fragments were purified from agarose gels using the LMP agarose method or using the Geneclean kit.

A gel slice containing the appropriate DNA fragment was cut out of a LMP gel and placed in an eppendorf tube with 200 μl of 0.2 M Tris, pH 8.0 and 1 μl of 10 mg/ml tRNA. The tube was heated to 65°C for 10 minutes (or until the gel melts). The solution was extracted with an equal volume of Tris saturated phenol and the aqueous phase was removed. The organic phase was then back extracted with 200 μl of H_2O and the aqueous phases combined. After two extractions with phenol/chloroform (1:1) and one extraction with chloroform the DNA was precipitated with EtOH and suspended in TE buffer.

In the Geneclean method (based on the data of

Vogelstein and Gillespie, 1979) the 2-3 volumes of NaI were added to the gel slice containing the DNA. The gel was heated to 55°C until the gel dissolved and then 5 μl of Glassmilk were added. After a 5 minute incubation on ice the Glassmilk was pelleted with a 5 second spin in the microfuge. The pellet was washed 3 times in NEW buffer (NaCl/EtOH/Water). After the last traces of NEW were removed 20 μl of TE were added and the tube was heated to 55°C for 3 minutes. The Glassmilk was again pelleted with a 30 second spin add the TE containing the DNA was removed.

2.8.3   DNA analysis

Southern blots were performed essentially as described by Southern (1975). Following electrophoresis the agarose gel was soaked in denaturing solution containing 1.5 M NaCl and 0.5 M NaOH for 45 minutes with gentle agitation and then neutralized in 1.5 M NaCl and 0.5 M Tris-HCl pH 7.6 for 45 minutes. The DNA was transfered by capillary action over 12 hours (as described by Ausubel et al., 1989) using 10 X SSC (contains 1.5 M NaCl, 0.15 M trisodium citrate). After the transfer was complete the nitrocellulose was air dried and then baked in a vacuum oven for 2 hours at 80°C. Prehybridization and hybridization were performed in 6X SSC, 5X Denhardt's solution (0.1 % Ficoll, 0.1 %

polyvinylpyrrolidone, 0.1 % BSA), 0.5 % SDS, 100 μg/ml salmon
testes DNA and 50 % formamide. Prehybridization was for 2-3
hours at room temperature. Hybridization took place over 12
hours at 42°C in the presence of 10^5-10^6 cpm of heat denatured,
^{32}P labelled probe. Blots were washed 3 times (15 minutes per
wash) in 2 X SSC at 42°C then dried and exposed to film.

2.8.4   RNA analysis

50 μl of RNA was mixed with 50 μl of a solution
containing 12X SSC and 40 % formaldehyde and heated to 60°C
for 15 minutes. After quick cooling the RNA was spotted on
nitrocellulose which had been presoaked in 10 X SSC using a
slot blot apparatus. The filters were baked in a vacuum oven
at 80°C for 2 hours. Prehybridization and hybridization was
carried out in essentially the same manner as described for
the Southern blots.

2.8.5   Maxam-Gilbert sequencing

Chemical sequencing was carried out essentially as
described by Ausubel *et al.* (1988). Only the "G" and "G and A"
specific reactions were carried out.

For the G specific reaction 100 000 cpm of single-end-
labelled DNA (see preparation of DNAseI footprint probes) was

diluted to 10 μl and mixed with 200 μl of DMS reaction buffer (containing 50 mM sodium cacodylate, pH 8.0 and 1 mM EDTA). The G specific modification reaction involved the addition of 1 μl of dimethyl sulfate (DMS) followed by a 4 minute incubation at 25°C. The reaction was stopped by adding 50 μl of DMS stop buffer (containing 1.5 M sodium acetate, pH 7.0, 1.0 M β-mercaptoethanol and 100 μg/ml *E. coli* tRNA) and 750 μl of -20°C 100 % EtOH and immersing the tube in a bath of dry ice/ethanol for 5 minutes.

200 000 cpm of the probe was diluted to 10 μl for the G+A specific reaction. Bases were modified with the addition of 25 μl formic acid in a 5 minute reaction at 25°C. This reaction was stopped with by adding 200 μl of hydrazine stop buffer (containing 0.3 M sodium acetate, pH 7.0, 0.1 mM EDTA and 25 μg/ml *E. coli* tRNA) and 750 μl -20°C 100 % EtOH and immersing in a dry ice/ethanol bath for 5 minutes.

The precipitated DNA from both was pelletted and washed twice with 70 % EtOH. DNA was resuspended in 200 μl H$_2$O and reprecipitated with 20 μl 3 M sodium acetate and 500 μl 100 % EtOH. Stand scission was catalysed with the addition of 70 μl 10 % piperidine and incubating 30 minutes at 90°C. Piperidine was removed by drying the sample under vacuum. The DNA was then suspended in 30 μl H$_2$O dried and then this step was repeated. The cleaved DNA was separated by SDS PAGE electrophoresis on a 7 M urea sequencing gel.

2.9   Analysis of Proteins

2.9.1   Protein determination by the Bradford method

The protein concentration of various samples was determined by the Bradford method (Bradford, 1976). 1-20 μg of protein was made up to 0.8 ml with ddH$_2$O and mixed with 0.2 ml of Dye Reagent Concentrate. The OD$_{595}$ of each sample was measured and plotted versus the concentration of a standard (usually BSA).

2.9.2   SDS PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide electrophoresis was performed as described by Laemmli (1970) with the modifications described by Sambrook et al. (1989).

2.9.3   Immunoblot analysis

Western blots were performed essentially as described by Burnette (1981). Proteins were separated by SDS PAGE electrophoresis and then transferred onto a nitrocellulose filter using a BioRad Transblot apparatus. The transfer was allowed to proceed for 24 hours at 100 mA in a buffer

After the transfer was complete the nitrocellulose was rinsed in TBST (Tris-buffered saline containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05 % Tween 20) and then blocked in TBS containing 1 % BSA for 30 minutes with gentle agitation. The nitrocellulose was then transferred into a solution containing TBST and the appropriate primary antibody (usually diluted 1:200 to 1:2000 times). The filter was washed 3 times in TBST for 10 minutes each. The nitrocellulose was then transferred into TBST containing the appropriate secondary antibody (usually Goat-anti Mouse IgG Alkaline Phosphatase conjugate (BioRad)) diluted 1:5000 and allowed to incubate for 30 minutes at room temperature. The filter was then again washed 3 times in TBST. The nitrocellulose was blotted dry and transferred into the colour development solution which contained 330 μg/ml NBT, 165 μg/ml BCIP, 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. The reaction was allowed to proceed until the colour development was deemed sufficient.

2.10 Analysis of Protein-DNA Interactions

2.10.1 Mobility shift assays

The procedures used in the protein-DNA mobility shift assays were similar to those described by Fried and Crothers (1981) and Garner and Revzin (1981) as modified by Strauss and Varshavsky (1984). The standard DNA binding reaction was carried out in a 15 μl volume containing 10 mM HEPES, pH 7.9, 0.5 mM DTT, 0.2 mM EDTA, 60 mM KCl, 5 μg BSA, 4 μg competitor DNA (a 1:2 mixture of poly(dIdC)poly(dIdC):salmon testes DNA). HeLa nuclear extracts, rat liver extracts and/or purified proteins were added as indicated. Reactions were pre-incubated for 5 min at room temperature prior to added 0.15 pmoles of ^{32}P end labelled, synthetic duplex probe. The DNA probe most commonly used corresponded to the promoter proximal TAATGARAT element from the HSV-1 ICP0 gene (preparation and labelling described above). Binding reactions were incubated at 30°C for 20 minutes. 1 μl of loading buffer (30 % glycerol, 0.1 % Bromophenol Blue and 0.1 % Xylene Cyanol) was added to each reaction and the complexes were resolved on 3.5 % polyacrylamide gels (30:0.8 ratio of acrylamide to bisacrylamide) in 0.25X TBE buffer at 4°C. Gels were pre-run for 3 hours at 200 V prior to loading samples.

2.10.2 Detection of SF activity using the mobility shift assay

Mobility shift assays for the detection of SF activity were carried out essentially as described above. Binding reactions were supplemented before the pre-incubation step with 1 to 4 μl of an S100 extract or fraction thereof (as indicated). Control reactions were supplemented with the same volume of the appropriate buffer solution. SF activity was determined by cutting out the regions corresponding to the VIC complex from the dried mobility shift gel and quantitating the radioactivity by Cherenkov counting using a Beckman LS3801 scintillation counter.

2.10.3 DNAse I footprinting assays

DNAse I footprint assays were carried out using labelled DNA templates isolated from plasmid DNA as described above. The procedure used was similar to that used by Galas and Schmitz (1978) with some of the modifications described in Ausubel et al. (1988). Binding reactions were performed in a 50 μl volume in the presence of 0.6 μg poly(dIdC)poly(dIdC) in buffer containing 25 mM Tris pH 7.9, 2 mM $MgCl_2$, 10 % glycerol, 0.5 mM EDTA, and 0.5 mM DTT. HeLa cell extracts or purified proteins were added as indicated. Reaction mixtures were pre-incubated for 5 minutes at room temperature prior to

the addition of approximately 20 000 cpm of the labelled probe. The binding reactions were incubated at 30°C for 15 minutes. The protein bound templates were digested with 0.3 units of DNAseI freshly diluted in 25 mM $CaCl_2$ in a 60 second reaction at room temperature. The digestion was terminated with the addition of 100 μl of Stop Buffer containing 1 % SDS, 100 μg/ml tRNA, 200 mM NaCl, 20 mM EDTA, and 200 μl proteinase K (added fresh). The mixture was incubated at 50°C for 10 minutes and the DNA was ethanol precipitated. DNA pellets were resuspended in 5 μl of formamide loading buffer containing 80% formamide, 10mM NaOH, 1 mM EDTA, 0.1 % Xylene Cyanol and 0.1% Bromophenol Blue and heated to 95°C and quick cooled before loading. The DNA was resolved on a 10 % polyacrylamide gel containing 7 M urea. The gel was run at approximately 60 W for 2.5 hours before being dried and exposed to film.

STRUCTURE-FUNCTION ANALYSIS OF VMW65

Since Vmw65 closely interacts with the cellular transcription machinery, a detailed understanding of its function could help elucidate not only the nature of coordinate expression of viral genes but may also provide important insights into the more complex regulatory circuits operating in eukaryotic cells. Therefore the objective of this line of research has been to investigate the mechanism of action by which Vmw65 specifically stimulates IE gene transcription. As little was known about Vmw65 (beyond its primary sequence) when this work began, the first step of this investigation involved a structure-function analysis to map functionally important domains of the protein. This work therefore required (i) the development of a series of assays by which specific activities could be measured and, (ii) a bank of mutated versions of Vmw65 to analyze for deficiencies of a particular function.

3.1 The Identification of Domains Required for the Transactivation Function *In Vivo*

3.1.1 The Transient transfection assay

The ability of Vmw65 to stimulate transcription is easily monitored with the use of a simple transfection assay (similar to that of Gaffney *et al.* (1985)). This consisted of co-transfecting Vero cells with Vmw65 (in the form of pMC1) and p175*cat*, a plasmid containing the chloramphenicol acetyl transferase (CAT) indicator gene driven by the ICP4 promoter. The level of CAT activity in the cell extract is an indirect measure of the ability of Vmw65 (or mutant thereof) to transactivate expression from an IE promoter. While the basal level CAT activity in cells transfected with p175*cat* alone is very low, in the presence of Vmw65 (pMC1) activity is enhanced 30 to 40 fold (Fig. 3.1.1). A similar enhancement has also be shown by other labs (O'Hare and Hayward, 1984; Gelman and Silverstein, 1987a). The Vmw65 mediated stimulation of transcription is specific for promoters containing at least one TAATGARAT recognition element (see Fig 5.3.1). The availability of this assay system therefore makes it possible to measure the ability of Vmw65 mutants to transactivate transcription and thereby, allows regions of the protein that are sensitive to mutation to be mapped. A dose response curve for transactivation was produced by transfecting increasing
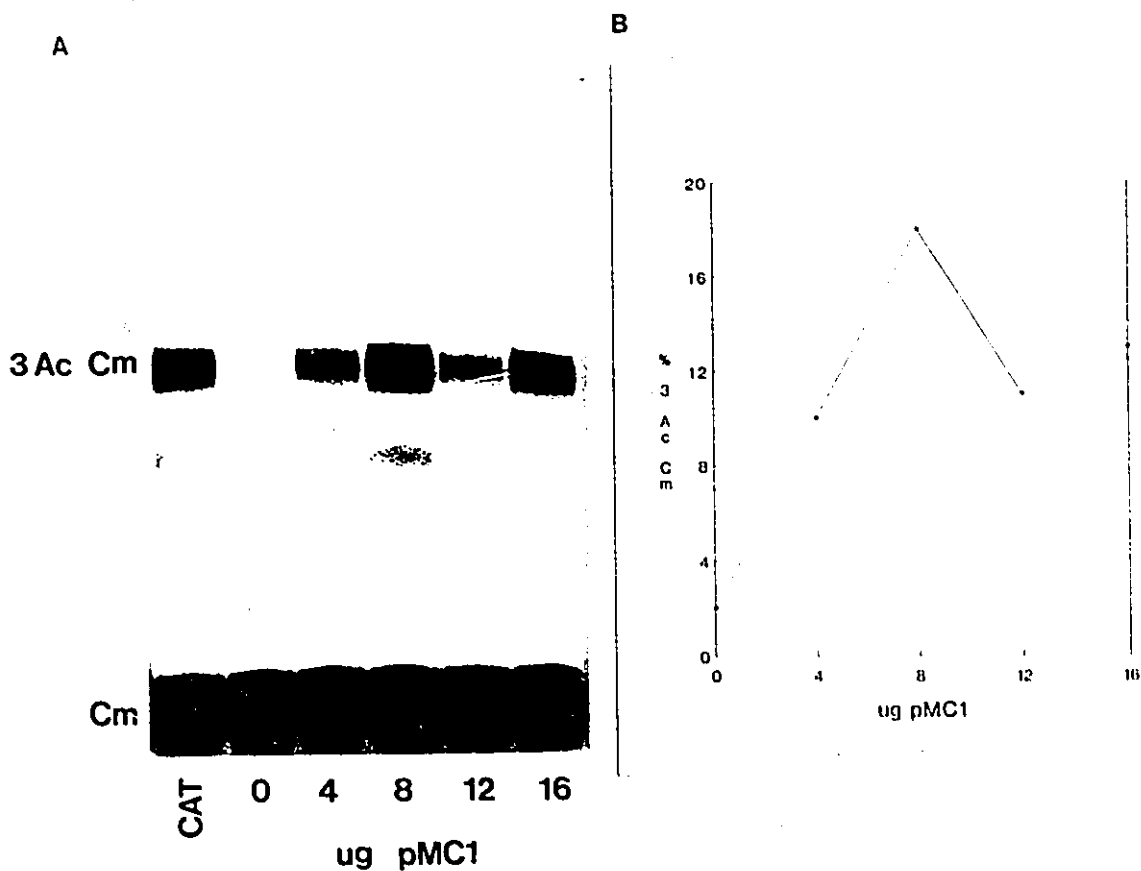
Fig. 3.1.1   Titration of pMC1. a) Vero cells were cotransfected with 4 µg of p175cat and 0, 4, 8, 12 and 16 µg of pMC1 (lane b-f, respectively). All transfections were normalized to 20 µg DNA with salmon testes DNA. Cells were harvested 48 hours post-transfection. As a control, the assay was carried out with commercially obtained CAT enzyme (lane a). The positions of chloramphenicol and its acetylated products are indicated. **b)** The above results were quantitated and plotted on a graph. The TLC plates were scraped and the % 3 Ac Cm was determined relative to the total cpm in each lane by scintillation counting.

amounts of pMC1 with a constant amount (2 μg) of p175cat (Fig.3.1.1). The results show that activation is linear with up to approximately 8-10 μg of pMC1 where CAT levels peak. The decline in CAT activity observed when transfections included greater than 10 μg of pMC1 is very likely a result of Vmw65 mediated squelching. Squelching can occur when a transactivator, in this case Vmw65, is present in high concentrations. Under these conditions the activator is believed to sequester essential transcription factors in solution and thereby actually reduce the level of transcription (Gill and Ptashne, 1988; Triezenberg et al., 1988). Another possible explanation is that the transfection of greater than 10 μg of pMC1 saturates the cells with promoter sequences. These sequences would act to bind up and deplete the cell of essential transcription factors. In light of this result, all subsequent transfections were optimized to maintain conditions within the range of linear response. This would ensure the proper quantitation of the transactivation efficiency of each of the mutants with respect to wild type Vmw65.

3.1.2 Construction of insertion, deletion and nonsense mutations in the Vmw65 gene

The mutational analysis of a protein initially requires the production of a series of relatively major

changes, spaced without bias, across the entire coding sequence of the corresponding gene. The results obtained from measuring the activities of these mutants can then subsequently be used to produce much more subtle groups of mutations specifically targeted to potentially sensitive regions. Linker insertion mutagenesis was selected as a suitable technique for the production of the first generation of Vmw65 mutants. Linker insertion mutagenesis involves the addition of short synthetic oligonucleotides into the region of DNA of interest. This method may be utilized to disrupt the conformation of structurally important nucleic acids (ie tRNAs), to disrupt and identify non-transcribed regulatory sequences (ie promoters/enhancers) or to insert codons (including stop codons) into the coding region of a gene (Heffron et al., 1978).

The plasmid pMC1, containing the entire transcribed region of the Vmw65 gene from HSV-1 strain 17 as well as 275 bp of its native promoter, was chosen as a starting point for mutational analysis (Fig. 3.1.2). This plasmid had previously been shown to be sufficient for enhancing the expression of genes driven by an IE promoter (Campbell et al., 1984). To construct the linker insertion mutants, pMC1 was randomly linearized by partial digestion with one of the following restriction endonucleases; HaeIII, RsaI, NruI. Each of these cleave DNA specifically to leave blunt ended fragments and

Fig. 3.1.2 Nucleotide and amino acid sequence of **Vmw65** as determined by Dalrymple *et al.* (1985). The entire coding region of Vmw65 (translated into the single letter amino acid code) and the 5' and 3' untranslated regions (that are present in the plasmid pMC1) are shown. The positions of the linker insertion sites are indicated (.) and appropriately designated. Mutants were named according to the restriction site into which the linker was inserted (H=*HaeIII*, R=*RsaI*, N=*NruI*) and the number of the first codon to contain linker derived sequences. Premature termination mutants are indicated by Am (amber) and the position of the stop codon. Restriction sites used in the construction of the various mutants are underlined. The sequences of the *BamHI* and termination codon linkers are indicated at the bottom of the figure. Adapted from Dalrymple *et al.* (1985).

BamHI linker
5' CCCGGATCCGGG

Stop linker
5' CTAGCTAGCTAG

(with the exception of *Nru*I) they each can potentially cleave the Vmw65 coding region many times (17, 12 and 2 cleavage sites, respectively, Fig 3.1.2). The yield of linearized plasmid was optimized by the addition cf ethidium bromide to predetermined concentrations (Shortle and Botstein, 1983). Ethidium bromide partially unwinds the covalently, closed, circular plasmid DNA by intercalating between the hydrophobic base pairs. The unwinding is limited by the closed nature of the DNA. Upon cleavage by an endonuclease, this stress is relieved, the DNA can completely unwind and a maximal amount of ethidium bromide is bound. The presence of the ethidium bromide there after inhibits any further cleavage and enhances the yield of linearized, single cut plasmid (Fig. 3.1.3). The concentration of ethidium bromide required appears to vary with the restriction enzyme used and with the number of restriction sites present.

A dodecameric linker containing a unique *Bam*HI site was ligated to the randomly linearized pMC1. The plasmids were then extensively digested with BamHI to remove multiple linkers, re-ligated and used to transform competent HB101 *E. coli*. Plasmids were screened for the presence of the *Bam*HI site which was subsequently mapped by restriction analysis on agarose (Fig. 3.1.4) and polyacrylamide gels. The family of insertion mutants (Fig. 3.1.5) were named according to the restriction site disrupted by the linker (ie H=*Hae*III, R=*Rsa*I
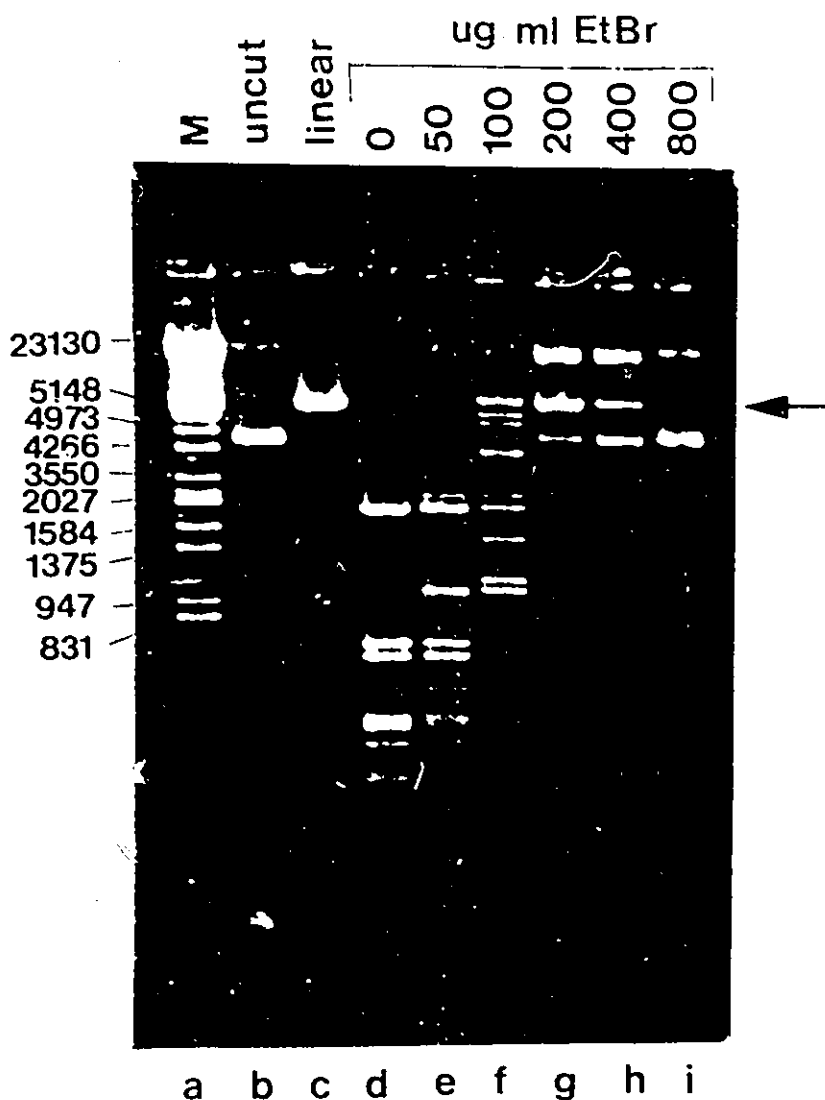
Fig. 3.1.3   The linearization of pMC1 by EtBr limited digestion. 1 μg of pMC1 was digested with 10 units of *Rsa*I for 1 hour at 37°C under optimal buffer conditions in the presence of 0, 50, 100, 200, 400 and 800 μg/ml EtBr (as indicated). The DNA fragments were separated on a 0.8 % agarose gel along with λ DNA cut with *Eco*RI and *Hin*DIII (lane a, fragment sizes in bp are indicated), uncut pMC1 (lane b) and pMC1 linearized with *Eco*RI (lane c). The DNA fragments were visualized, after soaking the gel in TBE containing 1 μg/ml EtBr, under a uv lamp. The position of linear pMC1 is indicated by the arrow to the right of the gel.
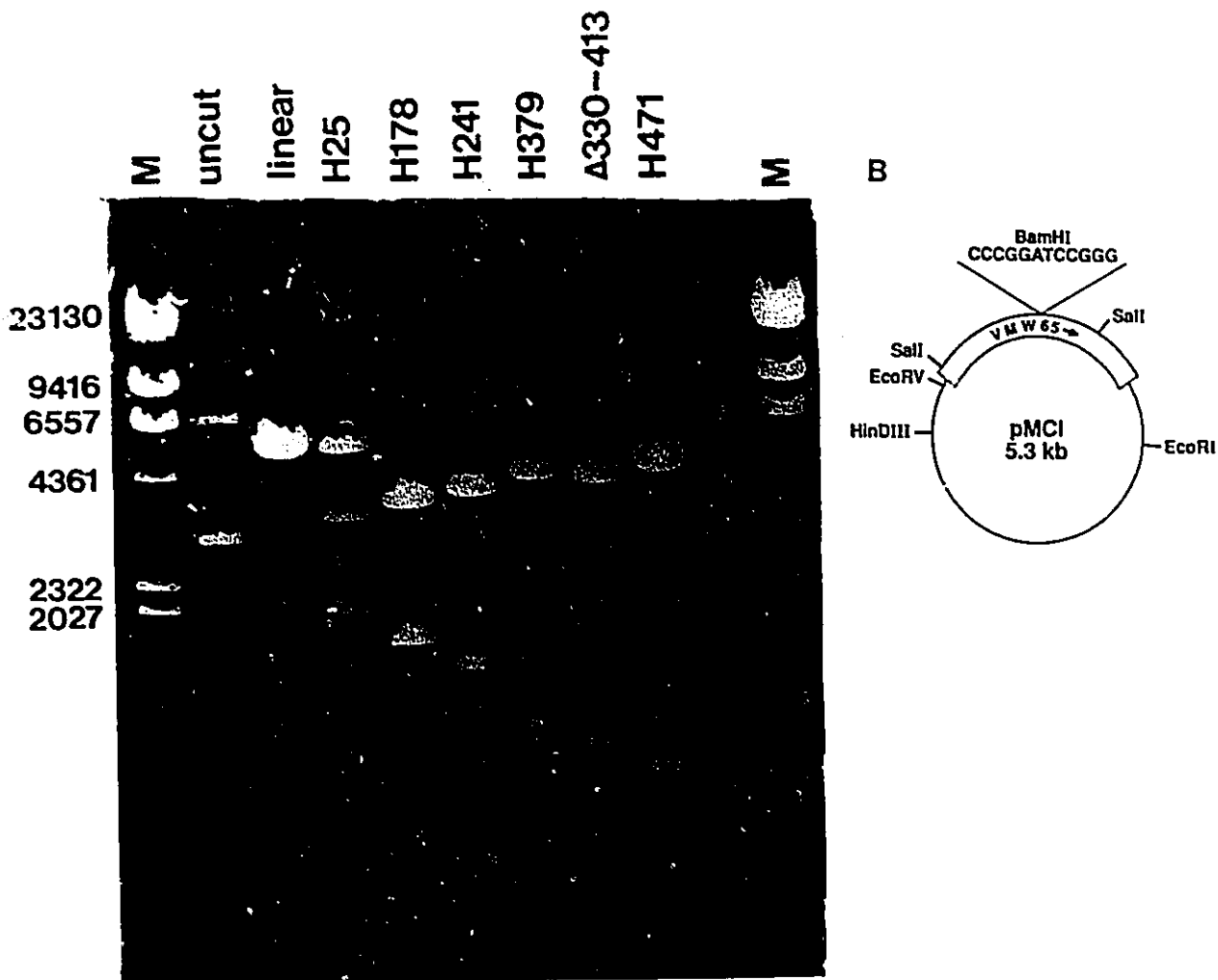
Fig. 3.1.4   Restriction analysis of several linker insertion mutants. A) The indicated mutant plasmids were double digested with *Eco*RI and *Bam*HI and the resulting DNA fragments were separated, along with uncut pMC1 (U) and pMC1 linearized with *Eco*RI (L), on a 0.8 % agarose gel. Marker lanes (M) contain λ DNA cut with *Hin*DIII (fragment sizes in bp are indicated). This gel illustrates how the position of the linker insertion sites (*Bam*HI sites) of the indicated mutants were mapped within the gene. B) A map of pMC1 showing select restriction sites and the *Bam*HI linker used for the insertion mutagenesis.
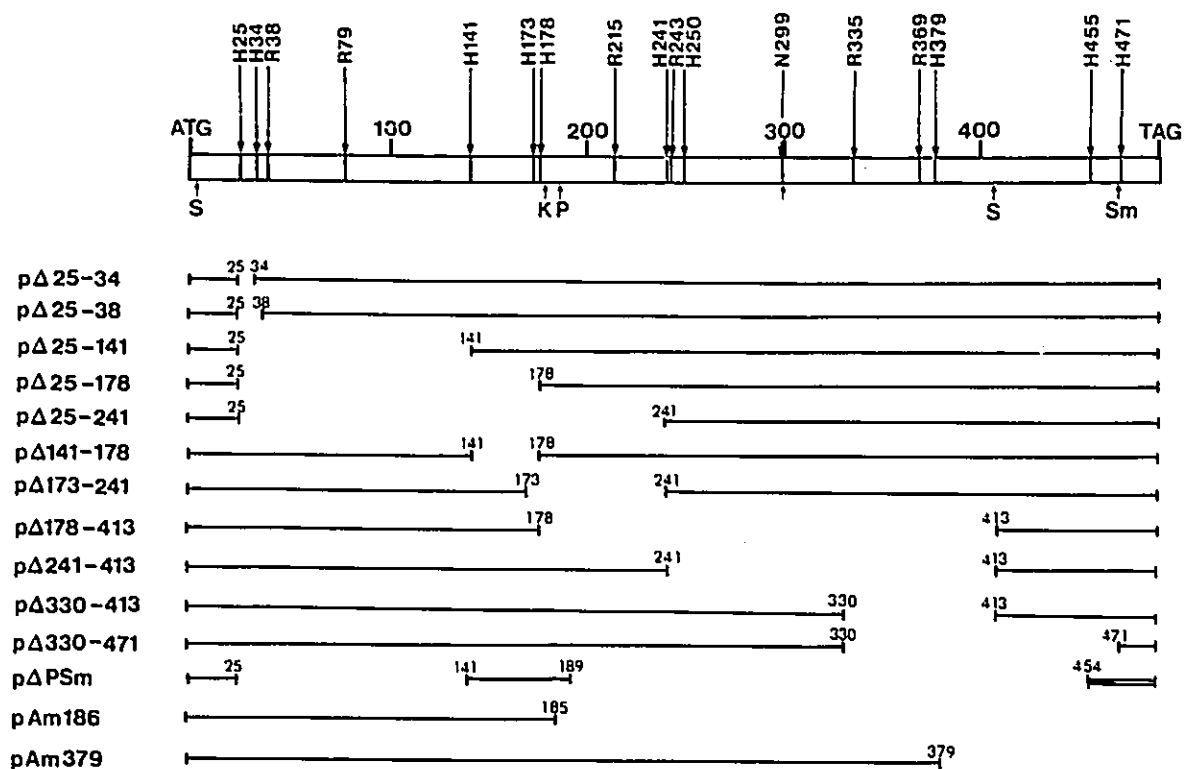
Fig. 3.1.5   Summary of Vmw65 mutants. A) Vmw65 is represented at the top of the figure with start and stop codons, numbered amino acids and a few key restriction sites indicated (S=SalI, K=KpnI, P=PvuII, Sm=SmaI). The position of each of the linker insertion mutants is shown at the top of the figure. **B)** The deletion and nonsense mutants are listed. The deleted regions are represented by gaps and the codons flanking each deletion are indicated. The double lined region of pΔPSm represents amino acid sequence unrelated to Vmw65 that is generated through a frame shift resulting from the PvuII-SmaI collapse.

and N=*Nru*I) and with respect to the first codon that contains nucleotides derived from the linker. Thus, H25 contains the *Bam*HI linker inserted in a *Hae*III site located 25 codons downstream of the initiator ATG (see Fig. 3.1.2).

In-frame deletion mutants were constructed by joining pairs of appropriately selected insertion mutants by virtue of their *Bam*HI sites. These mutants were named according to the first and last Vmw65 codons which flank the deleted region (Fig. 3.1.5). Deletion pΔPSm combines deletion mutant pΔ25-141 with a *Pvu*II-*Sma*I collapse. Mutant pAm379 was constructed by inserting the synthetic oligonucleotide (AB164), containing an amber stop codon (TAG) in all three reading frames, so that the encoded protein would be truncated after amino acid 379. Dr. J. P. Capone generated pAm186 by changing serine 186 (TCA) to an amber (TAG) stop codon using site directed mutagenesis.

3.1.3　Analysis of the ability of the linker insertion mutants to transactivate transcription

At the protein level, the insertion of 4 amino acids can reduce activity in two different manners; ideally, the insert will disrupt and thereby identify a functional domain, alternatively, the functional groups inserted may have secondary effects on protein stability, conformation or cellular localization. The type of amino acids inserted in each mutant is dependent upon the orientation of the original

restriction site used with the reading frame of the gene. Therefore, using a dodecamer, there are five different peptide sequences that could be added.

Each of the linker insertion mutants were tested for their ability to transactivate 175cat transcription relative to wild type Vmw65 (Fig. 3.1.6). A large range of activities were observed, from completely inactive mutants (ie pH178) to mutants with activity comparable to (ie pH455) or in excess of (ie pH241) wild type. The level of transactivation exhibited by each of the mutants tested is listed in Table 3.1.1, with wild type Vmw65 arbitrarily set at 100 %. Because of the inherent variability of the CAT assay and the efficiency of transfection, the mutants were divided into three categories. Those mutants showing activity greater than 40 % of pMC1 (wild type Vmw65) were considered wild type; mutants showing between 10-40 % activity were considered slightly affected; and those mutants exhibiting less than 10 % activity were considered inactive. Furthermore, experiments were repeated several times and total protein concentrations of the cell extracts were normalized in each case.

By the criteria stated above, several regions of the Vmw65 gene appear to be very sensitive to insertional mutagenesis. These include the sites H178, R215, R335, R369 and H471. Insertions into sites R79, H141, N299 and H379 were slightly defective while the remaining mutants displayed
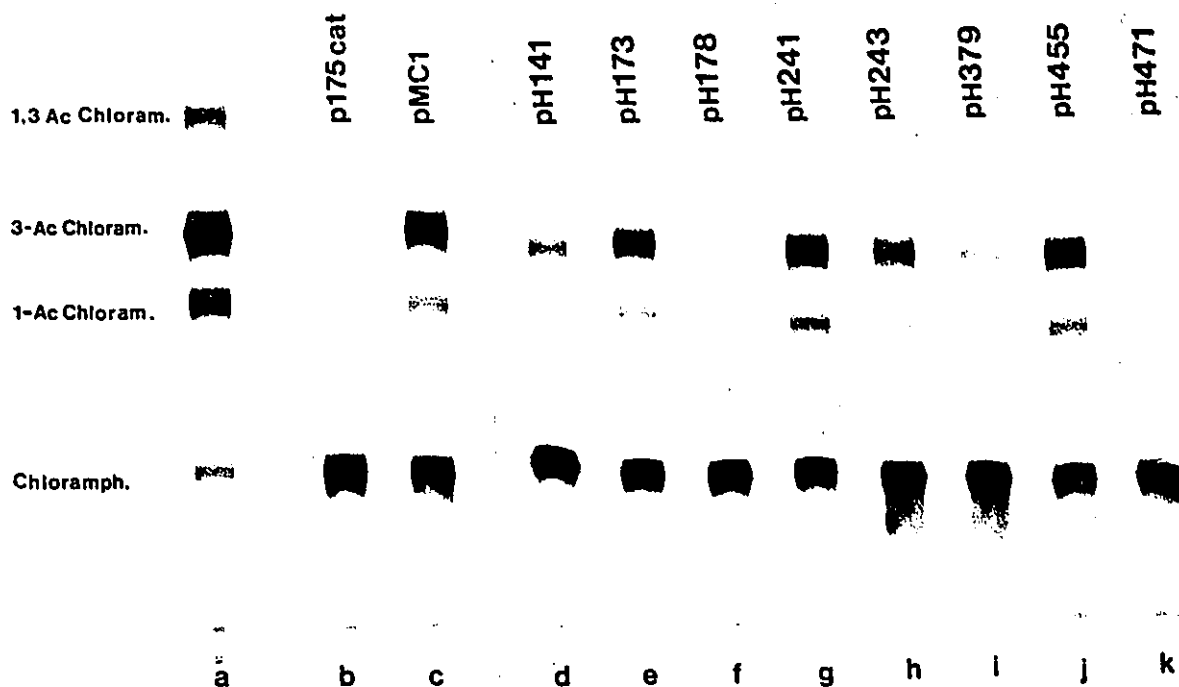
Fig. 3.1.6   Transactivation of 175cat by various Vmw65 mutants. Vero cells were transfected with 2 μg p175cat alone (lane b) or with 6 μg of pMC1 or mutant as indicated (lanes c-k). As a positive control the assay was also carried out with commercially obtained CAT enzyme (lane a). The positions of chloramphenicol (Cm) and the three acetylated derivatives are indicated.

Summary of activities of linker insertion mutants

Mutant[a] plasmid	Insertion site[b]	Amino acids inserted[c]	Transactivation[d] (% 3 Ac-Cm)	
pMCl	—	—	100.0 ± 17.4	(8)
pH25	73	RIRA	42.1 ± 17.5	(3)
pH34	100	RIRA	64.1 ± 18.8	(4)
pR38	112	SRIRD	68.1 ± 19.1	(5)
pR79	234	PGSG	34.9 ± 6.1	(3)
pH141	421	RIRA	22.2 ± 6.9	(4)
pH173	517	RIRA	77.8 ± 13.4	(4)
pH178	532	RIRA	<1	(4)
pR215	643	SRIRD	<1	(5)
pH241	721	RIRA	266.0 ± 82.5	(6)
pR243	727	SRIRD	40.3 ± 3.8	(5)
pH250	747	PGSG	57.8 ± 16.8	(4)
pN299	896	PDPG	38.2 ± 9.0	(3)
pR335	1003	SRIRD	<1	(5)
pR369	1114	PGSG	<1	(5)
pH379	1136	PDPG	10.5 ± 3.4	(4)
pH455	1362	PGSG	94.7 ± 38.3	(4)
pH471	1411	RIRA	<1	(4)

Table 3.2.1   Activities of the linker insertion mutants.
[a]Nomenclature of constructs is described in Fig. 3.2.5.
[b]Sequence numbered according to Dalrymple et al.(1985).
[c]Single letter aa code is used.
[d]Transactivation is measured as the % of 3 Ac-Cm produced (± the standard error of the mean) relative to wt pMC1 which was arbitrarily set at 100 % after subtraction of the basal level activity (5 ± 2 %). The values given are an average of 3-8 independent experiments, as indicated in parentheses.

activities approaching wild type. One exception was H241 which
exhibited an ability to enhance expression 2.5 fold greater
than wild type Vmw65. The significance of such a fortuitous
mutation is not clear but further studies have suggested that
this mutant may be altered with some respect to protein-DNA
complex formation (see later). From the data there is no
apparent correlation between the identity of the amino acids
inserted and the resulting activity of the mutant. However, it
is not possible to say that the identity of the residues did
not effect activity in each individual case. It is also
possible that the presence of the insert could effect mRNA
stability. Attempts were made to measure Vmw65 transcript and
protein levels in transfected cells in order to compare and
correct for expression levels. However, neither mRNA nor
protein was detected in any case. Therefore one must view the
results with these facts in mind.

From this crude mutational analysis there appear to be
at least three distinct regions of Vmw65 in which the inactive
mutations are clustered and a larger area of the protein that
can tolerate the addition of inserted amino acids (Fig.
3.1.7). These results are in general agreement with those of
Ace et al. (1988) who carried out similar experiments on
Vmw65. There are, however, two exceptions. In this work H173
and H471 were observed to be active and inactive,
respectively. The corresponding mutants, in8 and in17 in their
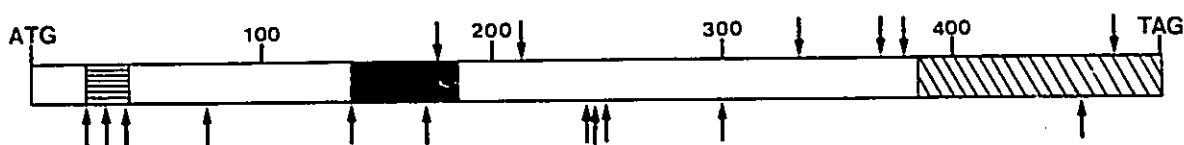
Fig. 3.1.7   Vmw65 mutational analysis summary. The coding region of Vmw65 is represented with the start and stop codons indicated. Amino acid number is shown at the top of the figure. Arrows directed upward represent sites where the insertion of the *Bam*HI linker had little or no effect on transactivation. Arrows directed downward represent sites where the linker insertion severely effected activity. The acidic carboxyl domain is indicated by the hatched region. The region represented by horizontal lines is non-essential for transactivation. The solid region corresponds to the sequences of Vmw65 that are sufficient to interfere with wild type transactivation *in vivo*.

study were inactive and active, respectively (Ace *et al.*, 1988). This may be a refection of cell types (they used BHK-21 cells) as every other parameter (including the amino acids inserted) were very similar if not equal.

3.1.4 Analysis of the ability of the deletion and nonsense mutants to transactivate transcription

The in-frame deletion mutants were also tested for their ability to stimulate CAT activity in transfected cells. These deletions range from small (8 aa) to very large (215 aa) and are spread across almost the entire protein (Fig. 3.1.5). Two small N-terminal deletions, Δ25-34 and Δ25-38, exhibited activities comparable to wild type Vmw65 demonstrating that this region of the protein is dispensable for transactivation (Table 3.1.2). This result is consistent with the linker insertion data which indicated that the N-terminus of Vmw65 could tolerate insertions with no significant effect on activity. All of the other deletion mutants that were tested show no significant ability to transactivate the 175cat gene. In some cases this is, perhaps, expected; for example, in cases where the deletion was constructed from one or two inactive insertion mutants, or in cases where the deletion is very large and extends across regions that are inactivated by insertion mutagenesis. Together these data indicate that Vmw65 does not contain large regions or domains that are dispensable

Properties of deletion and nonsense mutants of Vmw65

Mutant[a]	Linker insertion mutants used in construction	Amino acids removed[b]	Amino acids inserted[c]	Transactivation function[d]	Competition ability[e]
pΔ25-34	pH25, pH34	26– 33	RIR	+	ND
pΔ25-38	pH25, pR38	26– 38	RIRD	+	ND
pΔ25-141	pH25, pH141	26–140	RIR	–	+
pΔ25-178	pH25, pH178	26–177	RIR	–	–
pΔ25-241	pH25, pH241	26–240	RIR	–	–
pΔ141-178	pH141, pH178	142–177	RIR	–	–
pΔ173-241	pH173, pH241	174–240	RIR	–	–
pΔ178-413	pH178, pH413	179–412	RIR	–	–
pΔ241-413	pH241, pH413	242–412	RIR	–	+
pΔ330-413	pH330, pH413	331–412	RIR	–	+
pΔ330-471	·pH330, pH471	331–470	RIR	–	+
pΔPSm	combination deletion	26–140; 190–TGA		–	+
pAm186	nonsense mutant	186–TAG	–	–	+
pAm379	nonsense mutant	380–TAG	–	–	+

Table 3.2.2   Activities of the deletion and nonsense
mutants.
[a]Nomenclature is described in Fig. 3.2.5.
[b]pΔPSm contains 22 unrelated C-terminal aa.
[c]Linker aa remaining after the deletion.
[d](+) 30-95 % of activity of pMC1; (-) <5 % activity of pMC1.
[e]The ability of a 2.5 molar excess of each mutant plasmid to
interfere with wt (pMC1) transactivation of 175cat. (-) <10%
of wt CAT activity observed, compared to transfections with no
competitor; (+) >90 % of wt CAT activity observed; (ND) not
determined.

for its transactivation function.

The two premature termination mutants were both unable to transactivate p175cat. The C-terminus of Vmw65 (deleted in Am379 and Am186) contains a proportionally large number of acidic amino acids (18 out of 78) and is therefore highly negatively charged. Acidic domains had previously been implicated in the transactivation function of the yeast proteins GAL4 and GCN4. The requirement of the acidic C-terminal region in Vmw65, as exhibited by the inactivity of Am379, may suggest that it also functions as a transactivation domain. The activities of the two insertion mutants in this region, H455 and H471 may be rationalized to support such a hypothesis. H455 contains an insert of 4 uncharged amino acids (PGSG) that have little effect on transactivation. H471 contains an insert of 4 amino acids (RIRA) two of which are charged. This mutation is completely defective for transactivity. The effect of this insertion therefore may be a result of a local charge disruption (ie the insertion of 2 positively charged amino acids in a negatively charged domain). Subsequent to this work, Sadowski et al. (1988) conclusively demonstrated that the C-terminal 78 amino acids of Vmw65 act as a potent transactivator of transcription. In addition they showed that this domain is modular in that it is functional when fused to a GAL4 DNA binding domain in the absence of any other Vmw65 sequences. Many labs have

subsequently investigated the Vmw65 acidic activation domain
(AAD) with regard to its structure (Zhu et al., 1990; Cress
and Triezenberg, 1991b; Donaldson and Capone, 1992; O'Hare and
Williams, 1992) and the interactions it makes with the general
transcription factors of the initiation complex (Berger et
al., 1990; Ingles et al., 1990; Kelleher et al., 1990;
Stringer et al., 1990; Lin and Green, 1991; Lin et al., 1991).
These results will be discussed in greater detail in following
chapters.

3.2   Deletion Mutants Can Act as Trans-Dominant Inhibitors of the Wild Type Transactivation Function

The specific transactivation of transcription though
an enhancer element such as TAATGARAT is thought to require
at least two recognition events. The interaction between the
transactivator and the DNA sequence provides the specificity,
and the interaction between the transactivator and components
of the initiation complex provides the impetus that stimulates
transcription. In the case of Vmw65, because it lacks the
ability to strongly bind to DNA on its own, the DNA binding
function is augmented by cellular factors that are required to
assist in complex assembly.

In theory, at high concentrations of Vmw65, some of

the cellular factors required for transactivation will become depleted and the system would no longer respond to the subsequent addition of Vmw65. If Vmw65 can interact with some limiting component independently of the other cellular factors then a decrease in transactivation may be observed upon addition of more Vmw65 as non-functional complexes containing limiting, essential factors are formed. This phenomenon is termed "squelching" (Gill and Ptashne, 1988; Berger et al., 1990; Tasset et al., 1990). In fact, this type of response is similar to what was observed in titration experiments with pMC1 (Fig. 3.1.1). If wild type Vmw65 could compete with itself for some limiting component in the transactivation assay, then perhaps some of the inactive Vmw65 mutants retain this ability in the absence of a transactivation function. It was conceivable that the extensive bank of transactivation defective Vmw65 mutants available may allow the localization of a domain that may function as a trans-dominant inhibitor of the wild type protein.

To test this theory, pMC1 (4 μg) and p175cat were co-transfected with a 2.5 molar excess of various mutants that were previously determined to be inactive in the transactivation assay. CAT activity was used as a measure of the ability of wild type Vmw65 to transactivate transcription. Under these conditions the equivalent amount of wild type Vmw65 would saturate the system (see Fig. 3.1.1). To control

for possible promoter and translation effects of the competing plasmids, the promoter dosage was normalized in each transfection with pΔSal. pΔSal was constructed by collapsing the 1.2 kb Sal fragment out of pMC1. This plasmid therefore contains the entire promoter region and the first 5 amino acids of Vmw65 followed by a 70 amino acid sequence that bears no relation to Vmw65. Co-transfections of 10 μg of pΔSal had no effect on the 175cat activity (Fig. 3.2.1, lanes a and b) or on the transactivation by 4 μg of pMC1 (lanes c and d).

The results (Fig. 3.2.1, Table 3.1.2) show that the deletion mutants Δ25-141, Δ241-413, Δ330-413 and Δ330-471 can each interfere with wild type Vmw65 transactivation when co-transfected in a 2.5 molar excess with pMC1. This would suggest that each of these constructs contain one or more intact domains of Vmw65 that are still capable of interacting with at least one limiting factor involved in some step of transactivation. Therefore, these mutants retain their ability to compete with wild type Vmw65 and a decrease in CAT activity is observed. Conversely, the deletion mutants Δ25-178, Δ25-241, Δ141-178, Δ173-241 and Δ178-413 have no effect on the stimulation of CAT expression by wild type Vmw65. Such a result can be explained by the deletion or disruption of a binding domain. When examined together, these data indicate that a specific region or domain of Vmw65 is capable of interfering with wild type transactivation. The
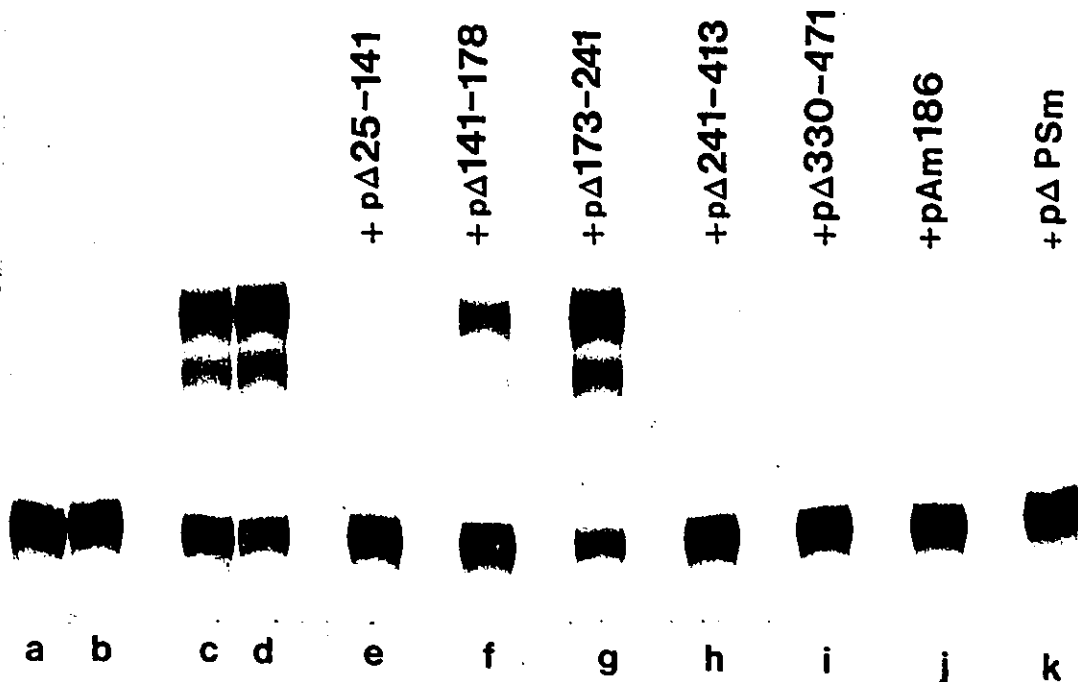
Fig. 3.2.1   Select Vmw65 deletion mutants interfere with wild type transactivation. Vero cells were cotransfected with 2 µg p175cat and 0 µg (lanes a and b) or 4 µg pMC1 (lanes c to k). 10 µg of each deletion mutant was added as indicated at the top of the figure. To control for promoter and translational effects of the deletion constructs pΔSal was added in lanes b and d. pΔSal contains the first 5 amino acids of Vmw65 followed by 70 unrelated amino acids.

region in question extends from amino acids 141-241. However, a similar result may be observed if the deletion of this region disrupted the stability of the transcript or the conformation or localization of the protein. This problem was addressed by testing the nonsense mutants in the competition assay. Both Am186 and Am379 were capable of interfering with wild type activity. This result narrowed the region of interest to amino acids 141-185, since both Δ25-141 and Am186 retained their ability to compete.

To further test the role of aa 141-185 the ΔPSm was constructed by digesting pΔ25-141 with PvuII and SmaI and removing the intervening sequences. The resulting mutant contains amino acids 1-25 and 141-189 of Vmw65 followed by 22 unrelated amino acids that are produced by a frame shift at the PvuII-SmaI junction. In co-transfections pΔPSm proved to be capable of competing out wild type transactivation in a dose dependent manner and a 3 molar excess of ΔPSm reduced activity to basal levels (Fig. 3.2.2). This positive result supports the assumption that there is a region between aa 141-186 that is capable of interfering with the function of wild type Vmw65; presumably though interactions with a factor required for transcription transactivation that is present in limiting amounts. The N terminal 25 amino acids of Vmw65 may also play a role in such an interaction as they are present in all of the constructs that have the ability to compete.
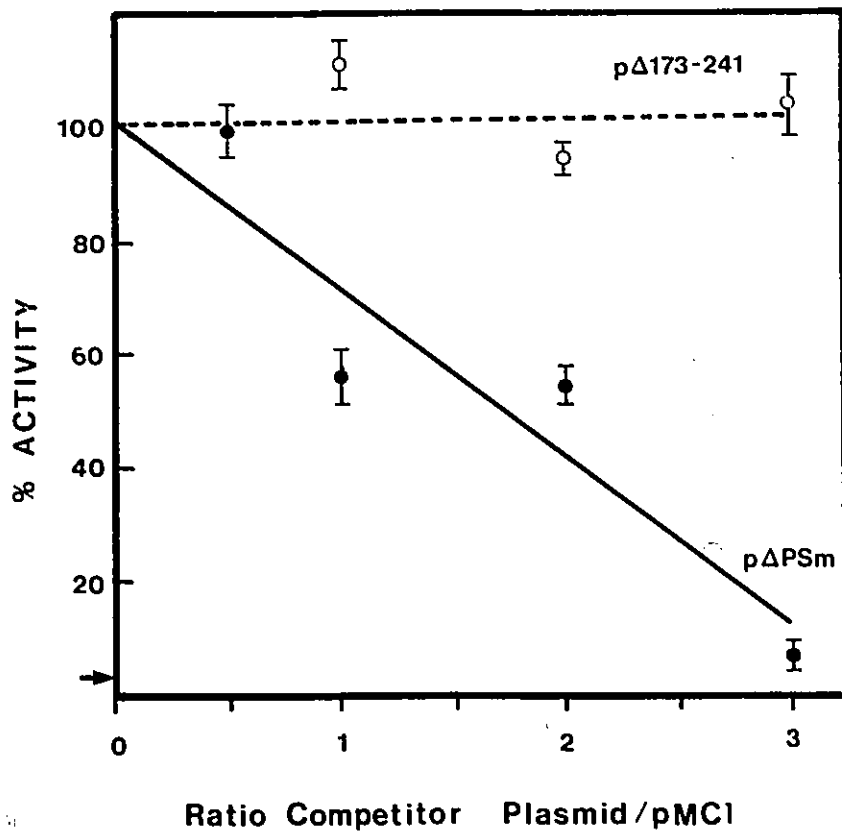
Fig. 3.2.2   Titration of pPSm. Vero cells were co-transfected with 2 μg p175*cat*, 4 μg pMCl and 0, 2, 4, 8 or 12 μg pΔPSm (solid line) or pΔ173-241 (dotted line). As a control, 2 μg p175*cat* was transfected alone. In all cases total DNA was normalized with salmon testes DNA. After 48 hours cells were harvested and CAT assays were performed. Results are plotted as percentages of the activity observed in the absence of competitor. Basal level *cat* expression is indicated by the arrow.

However, this region is definitely not sufficient for competition since it is also present in the defective mutants. Figure 3.2.2 also illustrates the inability of pΔ173-241 to affect transactivation at any concentration tested.

pΔPSm appeared to be incapable of reducing CAT activity below the basal level. Such a result suggests that the competition is at the level of activation and that pΔPSm is not interfering with normal pre-initiation complex formation by competing for general transcription factors.

To summarize, a region of Vmw65, potentially important for protein-protein or protein-DNA interactions and required in the stimulation of transcription, has been crudely mapped using a competition assay involving various inactive deletion mutants and wild type Vmw65. These experiments establish a starting point for subsequent, more stringent investigations with regard to Vmw65 and its interactions with other factors.

3.3 Requirements for the Assembly of the Vmw65 Dependent Complex

Vmw65 mediated transcription transactivation is conferred through cis-acting TAATGARAT enhancer elements and requires the presence of cellular factors including Oct-1. In order to correlate the ability of Vmw65 to activate transcription with its ability to associate, directly or indirectly with the TAATGARAT responsive element, a mobility shift assay was developed. This assay would facilitate the analysis of the Vmw65 dependent complex and permit the direct determination of the role of amino acids 141-185 and other regions in complex formation. In addition, the availability of such a system allows one to examine sequence specific protein-DNA complexes *in vitro* under controlled conditions. The advantages of the assay include its simplicity, speed and most importantly, its high degree of sensitivity which enables the detection of femptomolar amounts of DNA binding protein (Ausubel *et al.*, 1988). In this particular case the task was greatly simplified by our knowledge of the recognition sequence (TAATGARAT) which permitted the use of synthetic oligonucleotides in the assay. The actual sequence chosen for the majority of these studies is the promoter proximal TAATGARAT from the HSV-1 ICP0 gene. This element contains a consensus octamer binding sequence overlap and was also used in the experiments of Gerster and Roeder (1988). Versions of

this sequence were synthesized which contained point mutations in the octamer or the GARAT portions of the recognition sequence. A Type I TAATGARAT sequence from the ICP4 promoter which does not contain an octamer overlap and was also synthesized (see Fig. 3.3.2).

When incubated with HeLa cell nuclear extract the mobility of the ^{32}P-end labelled probe is significantly decreased on a 3.5 % non-denaturing gel (Fig. 3.3.1, lane a). The major protein-DNA complex that is formed contains the octamer binding protein, Oct-1 as previously demonstrated by others (Gerster and Roeder, 1980; O'Hare and Goding, 1988; O'Hare et al., 1988). The addition of an HSV-1 protein extract containing Vmw65 to the incubation results in the formation of another complex of decreased mobility (lane b). This complex has been designated IEC (Preston et al., 1988), C1 (Kristie et al., 1989) and VIC (Gerster and Roeder, 1988). Within this text the complex will be referred to as VIC, the Vmw65 induced complex or the Vmw65 dependent complex. The presence of Vmw65 in this complex is verified by supershifting VIC with the monoclonal antibody, LP1, that is specific for Vmw65 (lane c). The Oct-1 and Vmw65 dependent complexes form equally well on a type-I TAATGARAT element (ie one which lacks the complete octamer binding motif, lanes d-f).

The specificity of protein-DNA complex formation was tested using mutant versions of the TAATGARAT probe in the
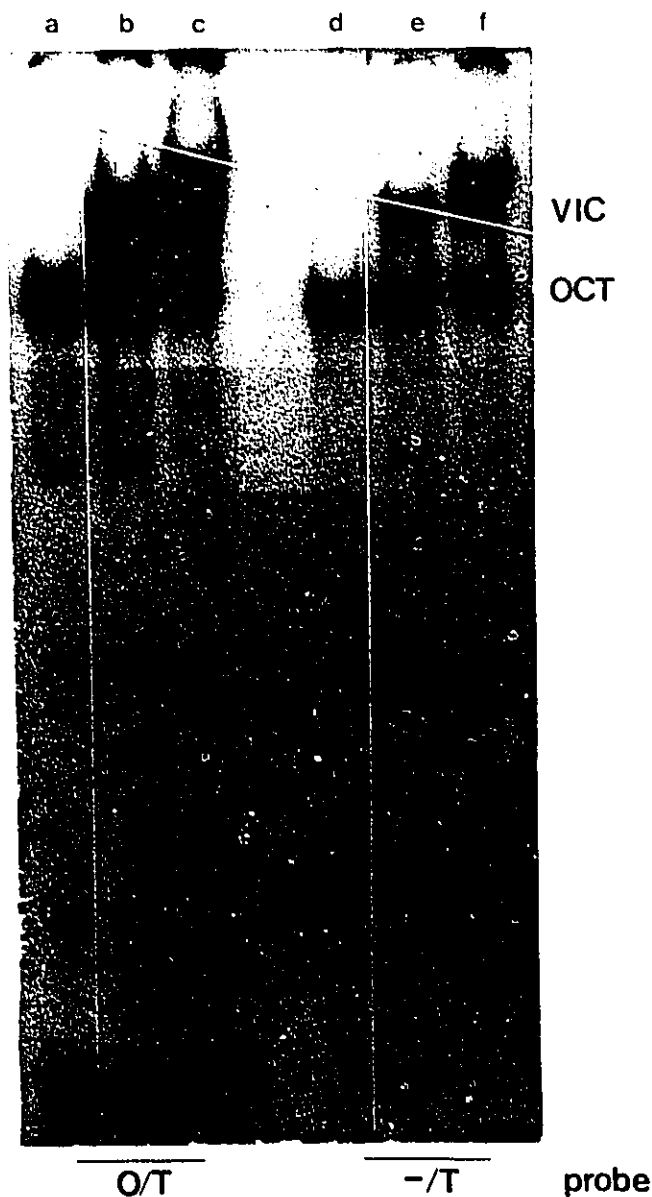
Fig. 3.3.1   Vmw65 dependent complex shifts the mobility of a TAATGARAT probe on a native gel. 8 μg of HeLa nuclear extract was incubated with a synthetic, double stranded DNA probe containing the IE0 promoter proximal ATGCTAATGARAT element (O/T) or a TAATGARAT element derived from the IE4 promoter (-/T). Binding reactions were supplemented with 0.5 μg of HSV virion extract containing Vmw65 (lanes b,c and e,f). 1 μl of LP1, a monoclonal antibody specific for Vmw65, was added to the reaction in lanes c and f. Protein-DNA complexes were separated on a 3.5 % non-denaturing gel. The positions of the Oct-1 and VIC complexes are indicated at the side of the gel.

mobility shift assay (Fig. 3.3.2). The Oct-1 complex could form when nuclear extract was incubated with a probe containing point mutations in the GARAT region (lanes c,d) as well as with wild type probe (lane a,b) but no complex was formed on a probe containing two mutations in the octamer binding sequences (lane e,f). In the presence of Vmw65, VIC was observed with the wild type probe (lane b) but not with the GARAT⁻ (lane d) or OCT⁻ (lane f) probes indicating that the Vmw65 induced complex is dependent upon the presence of the GARAT sequence as well as an octamer binding site. A second way in which specificity can be observed is though the ability of unlabelled (cold) sequences to compete with labelled wild type probe for complex formation. As indicated in Figure 3.3.3 a 50 fold excess of unlabelled wild type probe or a probe containing a mutation in the GARAT region (GARAT⁻) is capable of competing away both the Oct-1 and VIC mobility shifts. The same quantity of OCT⁻ probe, however had no significant effect on the formation of either of these complexes. Together, these data indicate that the GARAT sequence is not essential in the binding of Oct-1 to DNA but is required for the assembly of the Vmw65 dependent complex. This result suggests that another component of VIC recognizes and binds to GARAT in the presence of Oct-1 but weakly or not at all in the absence of Oct-1.

WT   GA⁻   OC⁻   Probe

VIC

OCT

a   b   c   d   e   f

WT   5'- ATGCTAATGATATTC

GA⁻   T CCG

OC⁻   C A C
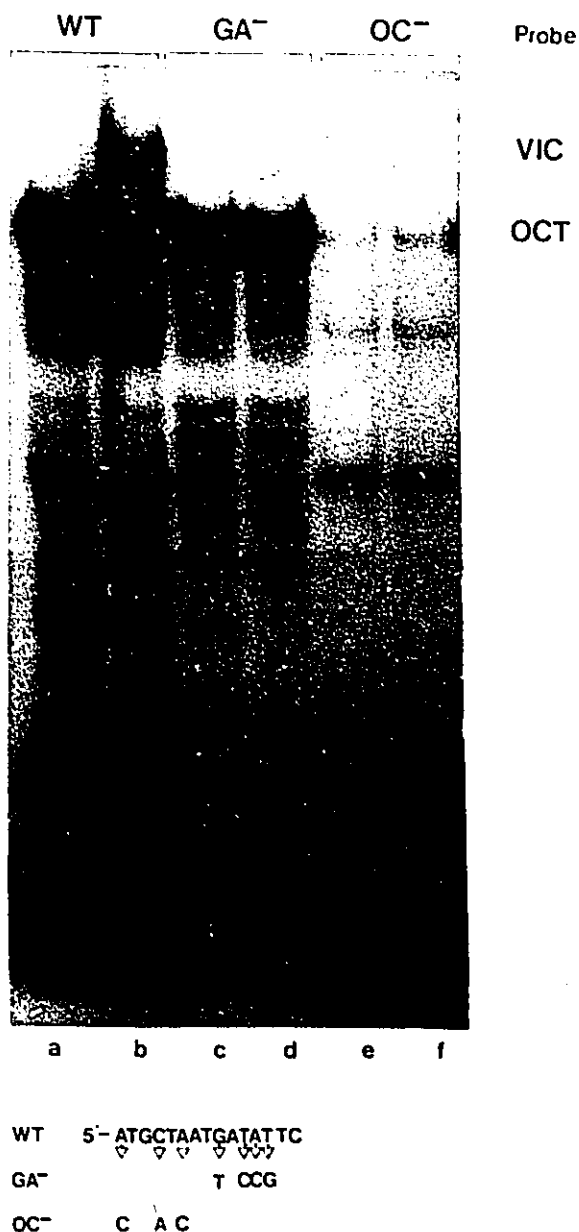
Fig. 3.3.2   VIC formation requires the complete TAATGARAT element. DNA probes containing wild type or mutated (GA⁻ and OC⁻) TAATGARAT sequences (as indicated) were incubated, under identical conditions with 8 μg of HeLa nuclear extract alone (lanes a, c, e) or with 0.5 μg of Vmw65 (lanes b, d, f). Complexes were resolved on a 3.5 % non-denaturing gel. The relevent sequence of the wt probe and the point mutations contained within GA⁻ and OC⁻ probes are indicated at the bottom of the figure.

Competitor labels (top): WT, GA⁻, OC⁻, WT, GA⁻, OC⁻

Right labels: VIC, OCT

Lane labels (bottom): a b c d e f g h
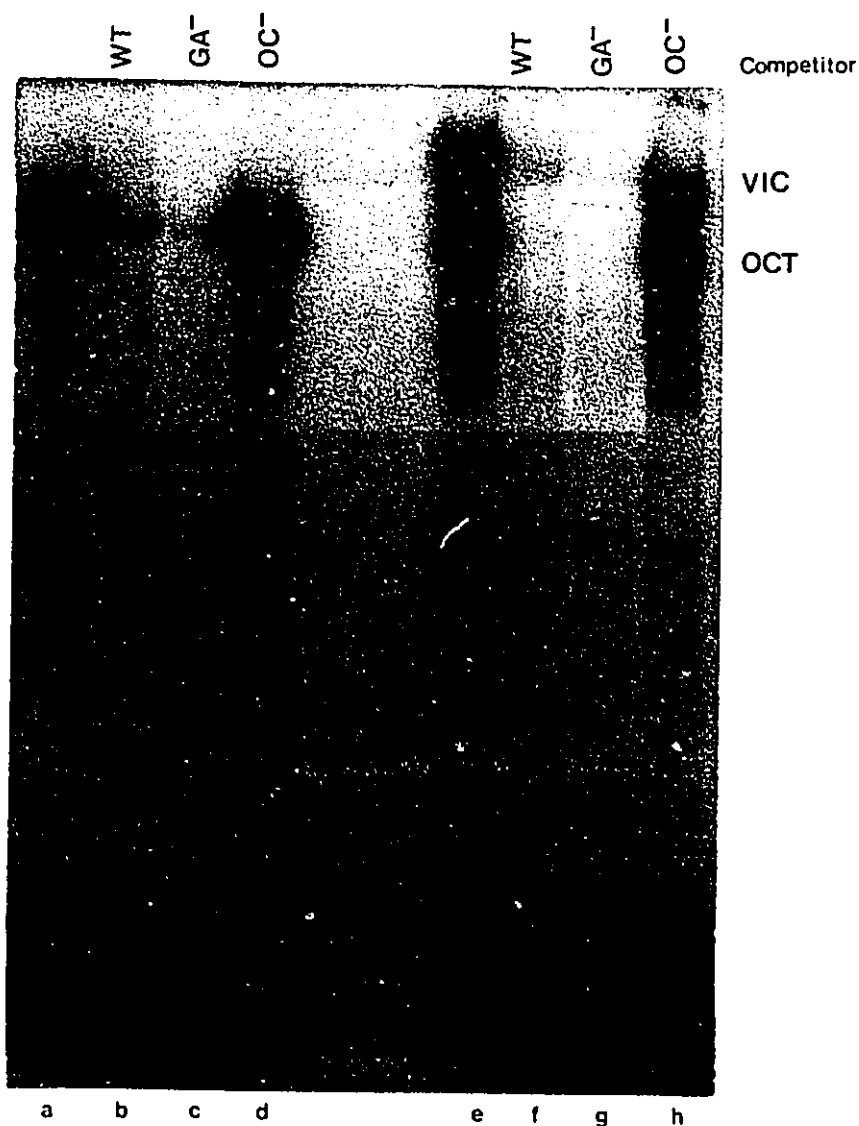
Fig. 3.3.3   Competition for complex formation with unlabelled probes. The TAATGARAT probe was incubated with 8.0 µg of HeLa nuclear extract alone (lanes a-d) or with 0.5 µg of Vmw65 (lanes e-h). A 50 molar excess of the appropriate unlabelled oligonucleotide was added during pre-incubation. The positions of the OCT and VIC complexes are indicated to the right of the figure.

3.3.1 Vmw65 produced in baculovirus or in *E. coli* as a fusion protein can form the Vmw65 dependent complex

Ideally, microgram quantities of relatively pure protein would be required to analyze the complex forming ability of each of the Vmw65 mutants. This could have been a formidable task because of the very low levels of expression that could be achieved with the tissue culture systems available. To circumvent this problem two new expression systems were established; in insect cells using the baculovirus infection system (Summers and Smith, 1987) and in *E. coli* using the protein A fusion system (Nilsson *et al.*, 1985). A baculovirus recombinant, AcNPV-65 expressing full length Vmw65 was constructed by Dr. J. Capone. Partially purified Vmw65 from insect cells extracts (Sf9, *Spodoptera frugiperda*) infected with this virus were incubated with HeLa cell nuclear extracts and tested for their ability to form VIC in the mobility shift assay (Fig. 3.3.4). The results clearly indicate that this was a feasible method for the over-production of Vmw65 and mutant versions thereof. However, this system had the disadvantage of being labour and time intensive. Each mutant would have to be sub-cloned into a transfer vector, transfected into SF9 cells, from which recombinant virus would then be plaque purified before large scale infections could be carried out. In addition the purification of sufficient Vmw65 from the crude extracts posed
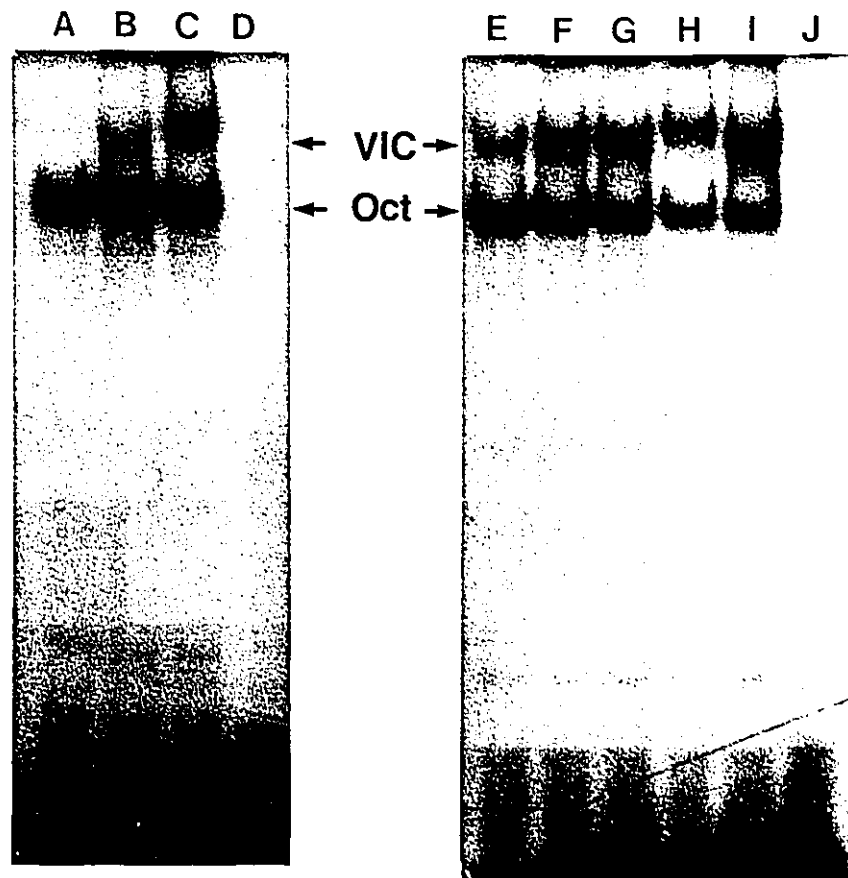
Fig. 3.3.4 Vmw65 produced in baculovirus supports VIC formation. An end-labelled DNA probe containing the TAATGARAT sequence was incubated with 8 μg of HeLa nuclear extract alone (lane a), with 0.5 μg of HSV-1 Vmw65 (lane b), with 0.5 μg HSV-1 Vmw65 and 1 μl of LP1 antibody (lane c) or with 0.1, 0.2, 0.5 μg of Vmw65 from AcVmw65 infected insect cells (lanes e to g). Lanes h and i are identical to g but also contained 1 μl of LP1 antibody or normal mouse serum, respectively. In the absence of nuclear extract, 0.5 μg of Vmw65 from HSV-1 (lane d) or from insect cells (lane j) does not detectably bind to the probe.

potential difficulties.

The protein A fusion system in *E. coli* provides a means to produce large quantities of protein together with a quick and simple method of protein purification. This, combined with the advantage of working with a bacterial system made it the system of choice for Vmw65 mutant over-expression. Of course a disadvantage of this system is the potential for the protein A domain interfering with the function of the Vmw65 sequences. To test the feasibility of this system sequences encoding amino acids 5-490 of Vmw65 were fused inframe behind the IgG binding domains of protein A. This construct, designated pRIT65, produced a protein, A65, that was capable of forming the Vmw65 dependent complex when incubated with a HeLa nuclear extract (see Fig. 3.3.6a,b). This result indicates that the protein A tail does not significantly interfere with complex formation and demonstrates that this fusion system is suitable as a means for the expression of protein to be utilized for the mapping of functional domains with the mobility shift assay.

3.3.2   Analysis of the ability of Vmw65 deletion mutants to form the dependent complex

The capability of a protein A fusion of Vmw65 to form the Vmw65 induced complex allowed the subsequent analysis of the bank of mutants. Select mutants were transferred from the

pMC1 plasmid into the pRIT2T system, over-expressed in bacteria and purified on IgG-Sepharose columns (Fig. 3.3.5). 0.5 μg of each of the deletion mutants was incubated with 8 μg of HeLa nuclear extract and run on a non-denaturing mobility shift gel. The results of some of the mutants are shown in figure 3.3.6a,b and are summarized in Fig. 3.3.9. The addition of protein A, produced using the pRIT2T system or commercially available protein A (Pharmacia), to the binding reaction had no effect on the Oct-1 complex and did not result in the formation of any additional protein-DNA complexes indicating that the carrier protein itself had no effect on the Oct-1-DNA complex formation (Fig. 3.3.6a, lanes g and m, respectively).

A65, the full length fusion protein, was capable of forming a VIC (lane c) that migrated slightly more slowly than VIC formed with viral Vmw65 (lane b), probably a result of the presence of the protein A tail. Asal is a truncated version of the fusion protein that is deleted of the C-terminal 79 amino acid acidic activation domain. This mutant forms a strong VIC complex in the mobility shift assay (lane d) indicating that complex binding domains of Vmw65 can function in the absence of the AAD. Thus Vmw65 contains at least two modular and independent domains; the AAD functions as a potent activator of transcription though interactions with the general transcription machinery; and within the remaining Vmw65
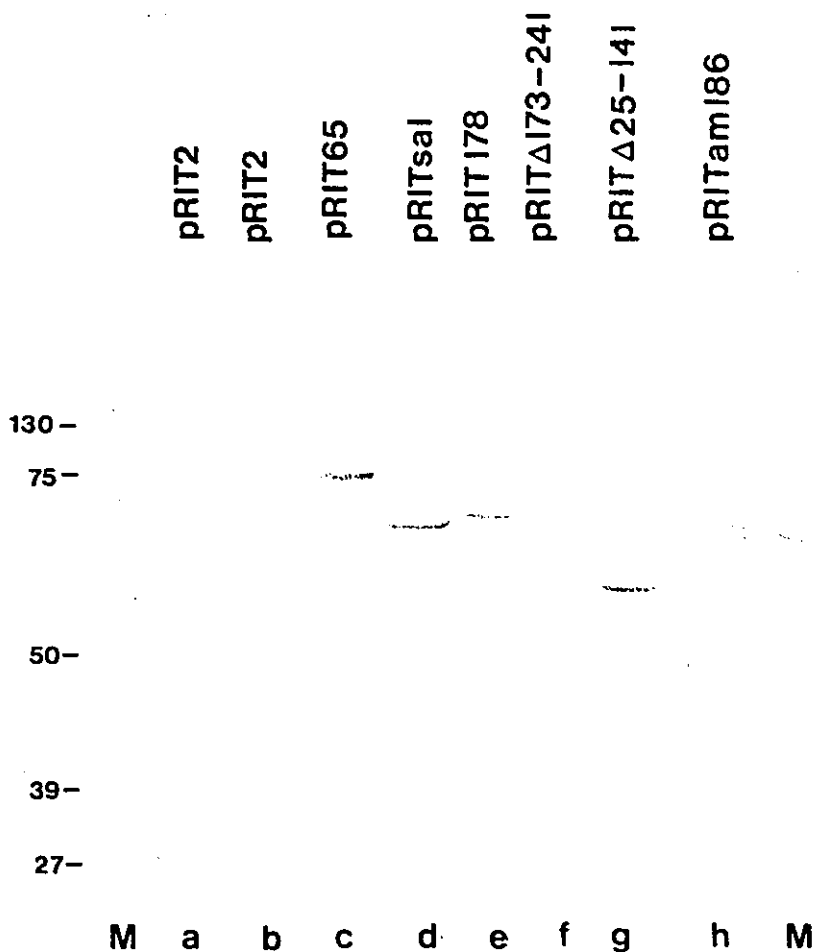
Fig. 3.3.5   Expression of select protein A-Vmw65 fusion proteins in *E. coli*. Bacterial cultures transformed with the indicated constructs were induced at 42°C and aliquots were resolved on an 8 % SDS PAGE gel. Proteins were transferred onto nitrocellulose. Fusion constructs were detected with rabbit anti-mouse IgG conjugated with horse radish peroxidase. Lane a is an uninduced control. The sizes of the Bio-Rad molecular weight markers are indicated in kDa to the left of the gel.
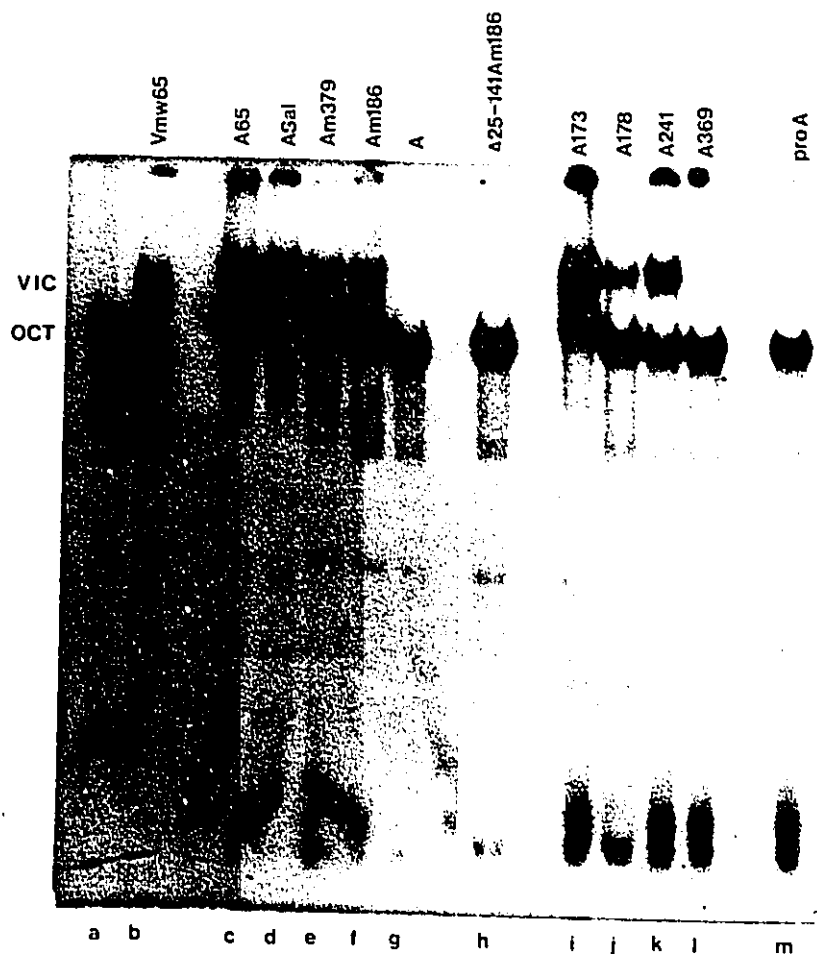
Fig. 3.3.6a Mobility shift analysis of the protein A-Vmw65 mutants. The labelled TAATGARAT probe was incubated with 8 μg of HeLa nuclear extract (lane a) and 0.5 μg of viral Vmw65 (lane b) or 2 μg of each of the indicated protein A fusion proteins (lanes c-l). Lane m contains nuclear extract and 2 μg of commercially obtained protein A. The protein-DNA complexes were resolved on a native gel. The positions of the Oct-1 and VIC complexes are indicated.
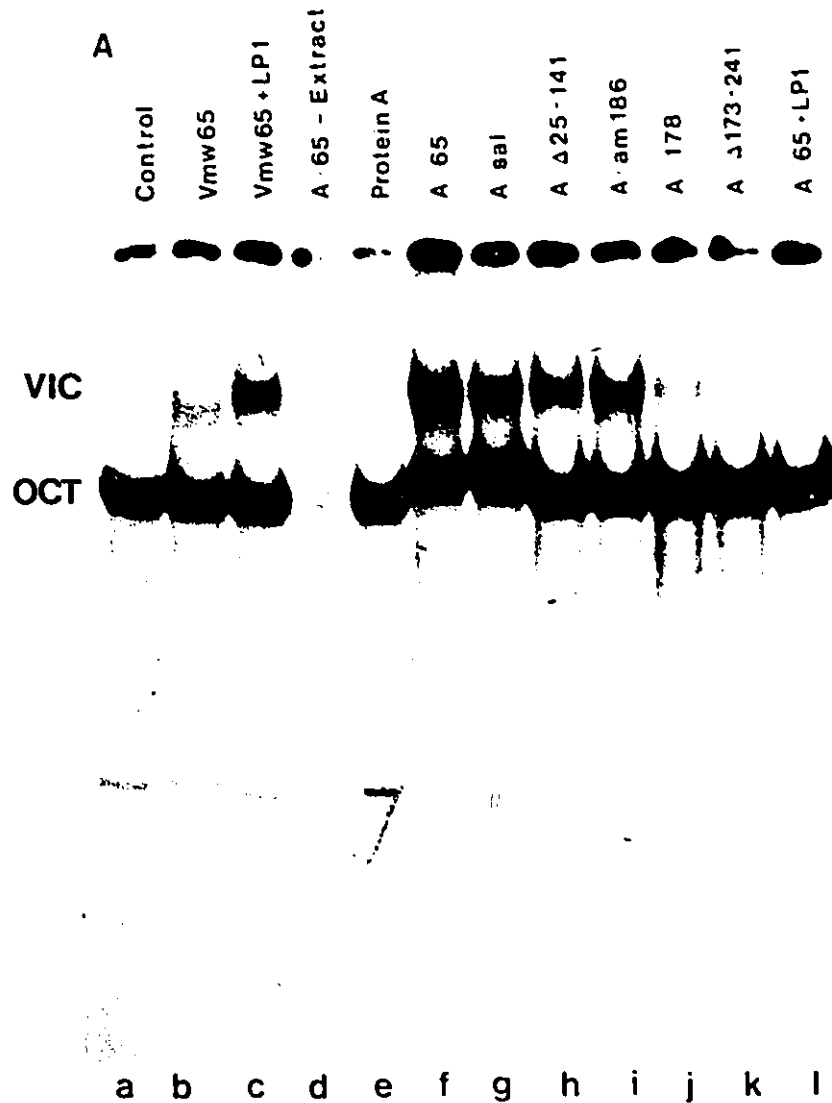
Fig. 3.3.6b   Mobility shift analysis of more protein A-Vmw65 mutants. The labelled probe was incubated with 8 µg of HeLa nuclear extract and the various additions as indicated at the top of each lane. 0.5 µg of Vmw65, expressed in insect cells, was present in lanes b and c. Protein A or protein A-Vmw65 fusion proteins were present in lanes d to 1. In lane d the nuclear extract was omitted. The positions of the OCT and VIC complexes are indicated.

sequences there exist protein/DNA binding domains that drive the formation of VIC, a complex that associates Vmw65 with the TAATGARAT recognition element. Further, the activation and complex formation domains are separable and each can impart its function to a heterologous protein.

The remaining mutants were used to further delineate the region(s) of Vmw65 required for VIC formation (data summarized in Fig. 3.3.9). The premature termination mutants, AAm379 and AAm186, are each capable of forming VIC although the specific activities are perhaps lower than those exhibited by Asal or A65 (lanes e and f). Therefore the region of Vmw65 between amino acids 186 and 411 is dispensable for complex formation even though it is sensitive to linker insertion mutagenesis in both the *in vivo* transactivation and the *in vitro* mobility shift assays. This result may suggest that mutations within this region can disrupt the function of Vmw65 by interfering with the structural integrity of this protein. Other researchers have reported that Vmw65 truncated at amino acid 388 is capable of driving VIC formation but truncations at amino acid 385, 316, 290, 189 or 181 eliminates this function (Greaves and O'Hare, 1989). One possible explanation of these conflicting data is that the amber stop codons inserted into constructs AAm379 and AAm186 are being suppressed and a read through product is responsible for driving VIC formation. However, no such products were ever

detected on Cromassie or silver stained SDS-PAGE gels nor by immuno blotting (Fig. 3.3.5). A more likely possibility is that the truncated versions of Vmw65 are inherently unstable but can be stabilized by the presence of the protein A domain in the fusion constructs. The ability of AAm379 and AAm186 to direct the formation of VIC indicate that they contain at least one domain that is involved in and sufficient for complex assembly.

In similar experiments deletion mutant AΔ25-141 was also shown to be capable of inducing complex formation (FIg. 3.3.6b, lane h). Thus by the process of elimination, amino acids 141-185 of Vmw65 appear to be necessary for the generation of VIC and therefore are capable of making (at least some of) the contacts involved in wild type VIC formation. Conversely, the mutants which have had this region deleted or disrupted, including AΔ25-241a, AΔ25-241b, AΔ141-178, and AΔ173-241, are all unable to drive the assembly of VIC. The combination deletion/truncation mutant AΔ25-141Am186 was constructed to test the ability of this region to form VIC *in vitro*. Mobility shift experiments with Δ25-141Am186 revealed that this construct induced VIC formation very poorly if at all (Fig. 3.3.6a, lane h). This result is somewhat surprising since the ability of AΔ25-141 and AAm186 to form VIC would lead one to predict that amino acids 141-185 are sufficient for complex formation. One explanation is that the

proximity of the protein A tail to this domain may interfere with its function. Another explanation may be that adjacent regions of Vmw65 play some role in the stabilization of this domain in its proper conformation. To eliminate these possibilities this region could be tested in the absence of the fusion peptide. The pGEX system, in which the GST tail can be specifically cleaved off, would be suitable for such an experiment. Finally, it is possible that essential contacts for complex formation are made both upstream and downstream of the 141-185 region. These may be redundant binding domains or different domains, one of which is sufficient in the absence of the other.

3.3.3 Analysis of the ability of select insertion mutants to form VIC

Several of the linker insertion mutants were transferred into the pRIT fusion system so that their ability to drive VIC formation could be correlated to their ability to transactivate transcription. Insertion mutants H173 (Fig. 3.3.6a, lane i) and H241 (lane k) formed the complex approximately as efficiently as "wild type" A65 while H178 (lane j) formed the complex poorly and R369 (lane l) not at all. The complex assembly defect of H178 and R369 explains the lack of activity of these mutants *in vivo*.

The ability of H241 to direct VIC formation is not

significantly different from A65. Therefore, the 2.5 fold enhancement of transactivation exhibited by H241 *in vivo* does not appear to be a consequence of the increased efficiency of this mutant to drive the assembly of VIC. This mutation must, therefore, positively affect transactivation at some other step in the process. This mutant is discussed in greater detail in a latter section.

In summary, these data indicate that the ability of Vmw65 to direct complex formation is necessary but not sufficient for its role as a transactivator of transcription.

3.3.4 Vmw65 contains an intrinsic DNA binding activity

A region of Vmw65 contained between amino acids 141-186 that is capable of interfering with wild type, Vmw65 mediated transactivation *in vivo*, also is important for complex formation *in vitro*. Together these results suggest that this region encompasses a domain that allows Vmw65 to interact with another factor that is involved in (and required for) the assembly of the Vmw65 dependent complex. This region, represented in the construct ΔPSm, is sufficient to interfere or compete with wild type activity *in vivo* suggesting that it is fully capable of binding to a limiting component and blocking complex formation. However, *in vitro* mobility shift assays with the construct AΔ25-141Am186 reveal that this

domain is incapable of efficiently driving complex formation on its own. One can draw several conclusions from these results. First, it would appear that regions of Vmw65 in addition to amino acids 141-185 are required to fulfil all of the necessary interacts involved in complex formation. This statement is supported by the results of Greaves and O'Hare (1989,1990) and Stern and Herr (1991). Second, amino acids 141-185 are sufficient to form at least one essential contact with a limiting component. Finally, this region can interfere with the wild type function by interacting with one or more limiting factors and blocking complex assembly and consequently, transactivation.

Vmw65 was initially reported to lack DNA binding activity (Marsden et al., 1987). In this study, extract from HSV-1 infected BHK cells was fractionated on a double stranded calf thymus DNA-cellulose column. Vmw65, detected by immunoprecipitation, showed no propensity to bind to this column. More recently, however, it has been demonstrated that Vmw65 is, in fact, a DNA binding protein that shows specificity for the TAATGARAT recognition sequence (Kristie and Sharp, 1990; Fig. 3.3.7). This interaction was probably overlooked in early studies because it is of such low affinity. Approximately 10 fold more Vmw65 is required to form a detectable Vmw65-DNA complex than is needed to produce VIC. The interaction is reportedly enhanced by the denaturation and

competitor   WT   GA⁻   OC⁻

| | | 20 | 50 | 100 | 20 | 50 | 100 | 20 | 50 | 100 |

A65   —   +   +   +   +   +   +   +   +   +   +

→

a   b   c   d   e   f   g   h   i   j   k
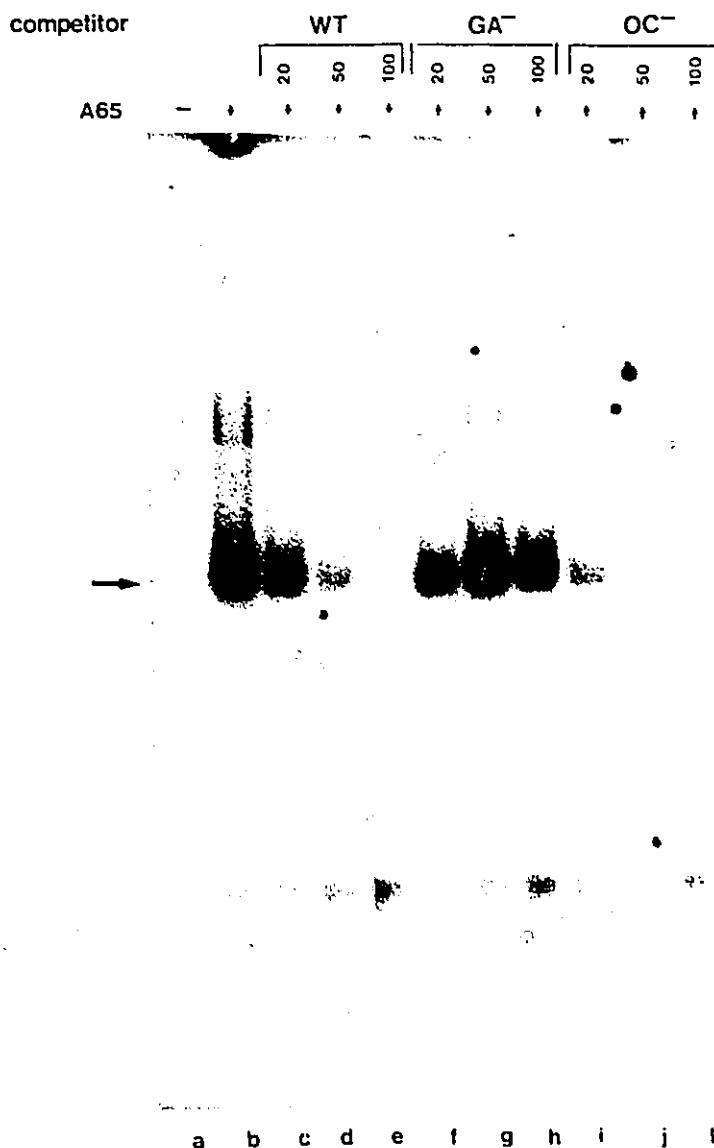
Fig. 3.3.7   Vmw65 binds specifically to the GARAT sequence. The labelled TAATGARAT probe was incubated alone (lane a) or with 10 μg of A65 (lanes b-i) and complexes were resolved on a native gel. Binding reactions included 20, 50 and 100 fold excess of unlabelled wt probe (lanes c-e, respectively), unlabelled GA⁻ probe (lanes f-h, respectively) or unlabelled OC⁻ probe (lanes i-k, respectively) as competitor. OC⁻ and GA⁻ contain point mutations in the octamer element and the GARAT sequence, respectively (see Table 2.1.1 or Fig. 3.3.2 for complete sequence information).

renaturation of Vmw65 (Kristie and Sharp,1990). The weakness of this association suggests that the direct binding of Vmw65 to TAATGARAT in the absence of other factors is not physiologically important and that transactivation occurs predominantly via the assembly of VIC. However, the ability of Vmw65 to specifically recognize a DNA sequence may play an important role in directing the transcription activation function to viral IE promoters as opposed to cellular promoters containing octamer sites. The specificity of this interaction is evident through the ability of wild type and OC⁻ (containing point mutations within the octamer motif) probes, but not GA⁻ (containing point mutations within the GARAT sequence) probe, to compete for complex formation (Fig. 3.3.7). Therefore, Vmw65 is a low affinity GARAT binding protein.

In order to map the Vmw65 DNA binding function to a specific domain, several of the Vmw65 mutants were tested for their ability to bind to TAATGARAT. The results indicate that the fusion proteins A65, Asal and AAm379 are each capable of forming a detectable complex with the TAATGARAT probe (Fig. 3.3.8). The fact that AAm186 is unable to detectably bind DNA even though it retains the ability to drive VIC formation may suggest this construct is capable of interacting with other components of the complex perhaps through protein-protein contacts. Recently, a 33 aa peptide representing Vmw65

O pro A ΔA65 ΔSal Am379 Am186 Δ173-241 Δ25-141 Am186 ΔI78

a   b   c   d   e   f   g   h   i

Fig. 3.3.8   DNA binding of Vmw65 mutants. 10 μg of each of the protein A-Vmw65 constructs, indicated at the top of the figure, were incubated with the labelled TAATGARAT probe and resolved on a native gel. As a control, the probe was incubated with no protein (lane a) or 10 μg of protein A (lane b).

protein A
fusion
constructs

1   100   200   300   400   490   V I C   D N A

S   S

A65 ——————————————————— 490   ++   +

ASal ——————————————— 411   ++   +

AAm379 ———————————— 379   ++   +

AAm186 ————— 185   ++   −

AΔ25-241   25   141 ————————— 411   ++   −

AΔ25-241a   25   241 ——————— 411   −   nd

AΔ25-241b   25   241 ————————— 490   −   nd

AΔ141-178   141   178 ————— 411   −   nd

AΔ173-241   173   241   411   −   −

AΔ25-141
Am186   25   141 185   −   −

A173 ——————————————— 411   ++   nd
173

A178 ——————————————— 411   −   −
178

A241 ——————————————— 411   ++   −
241

A369 ——————————————— 411   −   nd
369

pro A   −   −
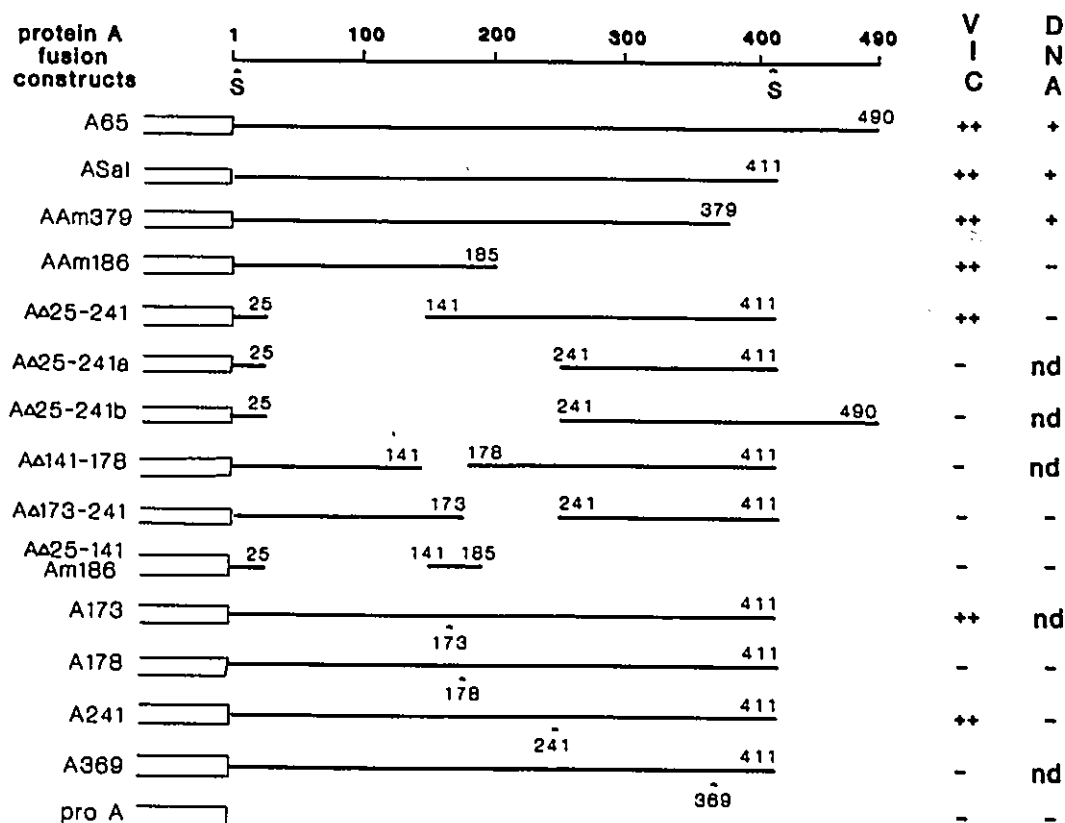
Fig. 3.3.9   Summary of the activities of the protein A-Vmw65 mutants in the mobility shift assay. The coding sequence for Vmw65 is indicated at the top of the figure. The open box represents protein A. The deletions within the Vmw65 sequence are represented by the gaps and the amino acids flanking each deletion are indicated. The first column to the right of the figure indicates the ability of each construct to drive VIC formation (ie strongly (++), weakly (+), or unable to form VIC (-)). The ability of each mutant to bind to DNA in the absence of host factors is summarized in the second column (ie (+) binds to DNA, (-) does not bind, (nd) binding ability not determined).

sequences from aa 170-202 was shown to bind to a TAATGARAT probe in a mobility shift assay (Stern and Herr, 1991). This report stated that the peptide-DNA complex could be competed away with non-specific DNA perhaps indicating the requirement of adjacent Vmw65 sequences for more stringent binding. This result, together with the data presented here, suggests that a low affinity DNA binding domain may be located in this region (aa 141-185) of Vmw65 that, in the context of the properly folded protein, may exhibit a specificity for the sequence, GARAT. Thus, the ability of pΔPSm to interfere with wild type transactivation *in vivo* may be a result of this truncated peptide binding GARAT and blocking further complex assembly.

Consistent with this theory, a closer look at the primary sequence in this region reveals that it contains a large number of basic residues, typical of DNA binding motifs. Specifically, this domain contains a series of arginine residues that can be aligned on one side of an amphipathic α-helix. A region of nine amino acids (ELRAREESY, corresponding to residues 160 to 168) is strongly conserved between Vmw65, its VZV homologue (ORF10, Dalrymple et *al.*, 1985) and its HSV-2 homologue (Cress and Triezenberg, 1991a). The conservation of this region may suggest that it plays an important role in the function of these proteins.

The structure of Vmw65 has been analyzed using the

Karplus-Shultz and Emini parameters for chain flexibility and surface probability and the Hopp-Woods and Eisenberg algorithms for hydrophobicity and hydrophobic moment, respectively (Fig. 3.3.10, Hopp and Woods, 1981; Eisenberg et al., 1982; Emini et al., 1985). These structural determinations predict that the region of Vmw65 spanning aa 150-240 is relatively flexible and hydrophilic, has a high α-helical content and a high probability of being located on the surface of the protein. These characteristics are typical of those expected of a domain involved in the recognition and binding of another factor.

3.3.5 General observations concerning mobility shift assays using the protein A fusions constructs

It is interesting to note that each of the fusion proteins capable of forming VIC produce a complex of identical size that migrates somewhat slower than that formed with wild type Vmw65. This would seem to suggest that differences in molecular weight of up to 35 kDa resulting from the deletion of Vmw65 sequences do not significantly effect the mobility of the complex. However, comparisons of the mobility of complexes formed with fusion proteins with those formed with HSV or baculoviral Vmw65 indicate that the addition of the 30 kDa protein A tail does notably decrease mobility. This apparent contradiction is most probably a result of the non-

Fig. 3.3.10   The predicted peptide structure of Vmw65. Plots were determined using the following methods; HW Hydrophilicity, Hopp and Woods (1981); Surface Probability, Emini *et al.* (1985); Flexibility, Karplus-Schultz (GCG manual); Antigenic Index, Jameson and Wolf (GCG manual); CF, Chou and Fasman (1978); GOR, Garnier *et al.* (1978). This plot was generated with the PLOTSTRUCTURE routine of the UWGCG software package.
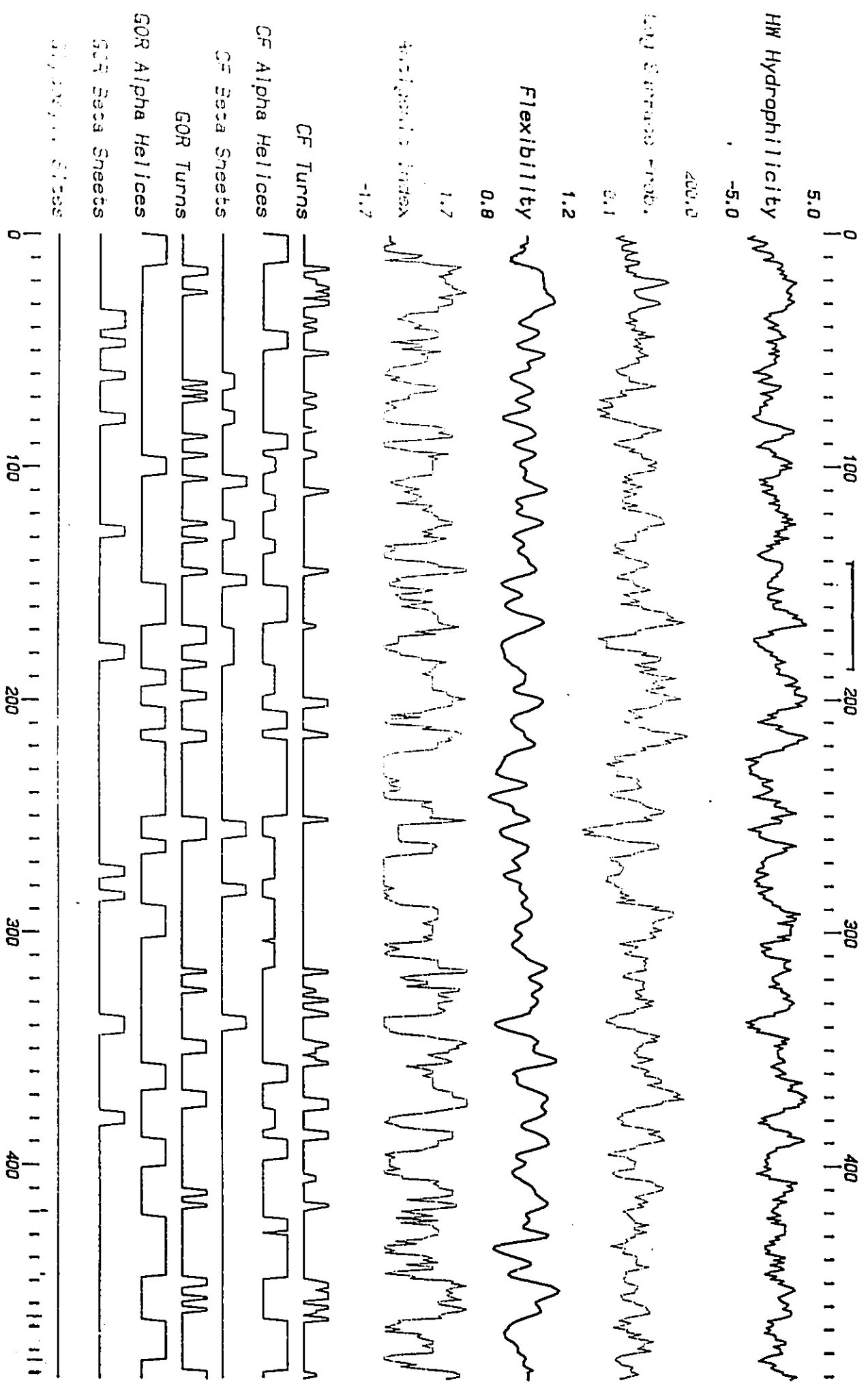
denaturing property of the mobility shift gel. Thus complexes are separated by virtue of their dimensional size and shape with less relevance to their molecular weights (Ausubel et al., 1988). Therefore, one would predict from this result that deletions within the Vmw65 region of the protein do not significantly change the dimensions of the complex. However, the protein A tail appears to extend out from the fusion protein causing a significant increase in the stokes radius of the complex. This is consistent with the observation that the protein A tail does not interfere with the complex forming domains of Vmw65. The fusion constructs cannot be supershifted with IgG and the addition of the antibody to the binding reaction appears to inhibit complex formation. The most likely explanation for this is that the immunoglobin interactions with the protein A region interferes in some manner with the Vmw65 binding function by sterically hindering complex assembly or by inducing a conformation change in Vmw65.

Some of the protein A-Vmw65 constructs consistently exhibited the ability to decrease the mobility of the Oct-1 complex. This effect was not observed with Vmw65 produced in transfected or infected eukaryotic cells although others have observed such effects (O'Hare and Goding, 1988; O'Hare et al., 1988; Greaves and O'Hare, 1989). Examples of this effect, described as a subshift, are evident in Figure 3.3.6. This effect was only evident with specific constructs and appeared

to be associated with a region of Vmw65 located downstream of amino acid 141 and upstream of amino acid 411. For example, it is evident in mobility shifts done with A65 and Asal, but not Am379 or Δ25-141. Interestingly, this region includes both the putative DNA and Oct-1 binding regions as delineated by Stern and Herr (1991). Significantly, there were no instances in which a subshift was observed with a construct incapable of forming VIC nor was this complex observed when Vmw65 from HSV-1 or baculovirus was utilized. It is possible that it may represent a partially assembled complex resulting from alterations in the affinity of certain of the recombinant fusion proteins for components of VIC. Oct-1 and Vmw65 have been reported to bind to TAATGARAT in a cooperative manner in the absence of VCAF-1 and other factors (Kristie and Sharp, 1990; Stern and Herr, 1991). The functional significance of this interaction is not clear. It may indicate that the complex can assemble via multiple pathways (with possibly different transcriptional outcomes) depending on the conditions within the cell.

3.4 Summary of the Structure-Function Analysis

In this work a series of Vmw65 mutants have been constructed in order to carry out a structure-function

analysis of this protein. Mutants were assayed for their ability to transactivate transcription and/or interfere with wild type mediated transactivation *in vivo*. In addition several of the mutants were tested for their ability to assemble into multiprotein-DNA complexes *in vitro*. Summarized together and in the context of data supplied by other researchers, these results provide a clarified picture of the functions and domains involved in Vmw65 mediated transactivation (Fig. 3.4.1).

All of the mutants that are incapable of forming a Vmw65 induced complex in a mobility shift assay are defective for transactivation *in vivo*, with no exceptions. Thus, complex formation would appear to be an essential step in the activation process. The converse, however, is not always true. Several of the transactivation defective mutants are not at all impaired in their ability to form VIC, suggesting that other interactions are required to form a functional complex. This is supported by the observation that some of these inactive mutants can interfere with the transactivation mediated by wild type Vmw65 *in vivo* presumably though interactions with other essential factors. This capacity to interfere with wild type transactivation was mapped to a region of Vmw65 contained within amino acids 141-185. Mobility shift experiments were utilized to show that this region is important in the assembly of the Vmw65 dependent multiprotein-

aa 160-168 of Vmw65
highly conserved
in ORF10 of VZV
(Dalrymple et al., 1985)
and its HSV-2 homologue
(Cress and Triezenberg, 1991)

ELRAREESY

synthetic peptide
corresponding to
aa 360-373
interferes with
VIC assembly
(Haigh et al., 1990)

```
1        100        200        300        400    490
```

N ▭ AAD C

synthetic peptide
corresponding to
aa 170-202
binds non-specifically
to DNA
(Stern and Herr, 1991)

aa 378-389
Oct-1 binding
domain
(Stern and Herr, 1991)

Fig. 3.4.1   Summary of Vmw65 interactions. The 490 amino acids of Vmw65 are represented by the open bar. The acidic activation domain (AAD) as well as the region containing amino acids 141-185 (hatched box) are shown. The location and importance of regions of Vmw65 that have been identified in other studies (see references) are indicated (these are discussed in greater detail in section 3.4 of this thesis).

DNA complex.

The fact that no large segments of the protein can be deleted without disrupting the transactivation function is perhaps indicative of the number of interactions that are required for Vmw65 to form a functional complex. These interactions can be divided between the two modular domains of this protein. The C-terminus AAD contacts components of the transcription pre-initiation complex in a manner which has yet to be clarified. The remaining 411 N-terminal amino acids are sufficient to drive the formation of the multiprotein, Vmw65 induced complex that specifically forms on the TAATGARAT enhancer element. In fact, at least three interactions are believed to be required for the assembly of this complex (see Fig. 3.4.1). Vmw65 binds strongly to a factor which has yet to be characterized (cloned) that has been designated VCAF-1 (also CCF, HCF and C1). It is not yet clear what regions of Vmw65 are required for this interaction and it is possible that several discontinuous sequences are involved (Xiao and Capone, 1990; Stern and Herr, 1991). A synthetic peptide containing amino acids 360-373 is capable of interfering with VIC formation but is has not yet been shown to bind a specific factor (Haigh et al., 1990). Vmw65 also interacts with Oct-1 in what appears to be a DNA dependent fashion. The Oct-1 binding domain has been mapped to amino acids 378-389 just upstream of the AAD (Stern and Herr, 1991). Finally, Vmw65

binds specifically, with low affinity to the GARAT region of the TAATGARAT sequence. This interaction is only observed on a mobility shift gel when the concentration of Vmw65 used is 10 fold higher than that required to form a detectable VIC. The Vmw65 DNA binding function can now be tentatively mapped to the middle of the protein. Stern and Herr (1991) have shown that a synthetic peptide containing Vmw65 sequences from amino acid 170 to 202 does indeed have non-specific DNA binding activity. The domain encompassing amino acids 141 to 185, as stated above, is capable of interfering with wild type Vmw65 mediated transactivation *in vivo* and is required to form a VIC complex *in vitro*. Computer modelling studies predict this region to form an amphipathic helix with a high probability of being located on the surface of the protein.

It is interesting to note that while the GARAT sequence is absolutely required to confer Vmw65 mediated transactivation, the stability of the Vmw65-GARAT complex is very weak in the absence of other factors. It is possible that upon interacting with Oct-1, VCAF-1 or another involved factor, Vmw65 undergoes a change in conformation that facilitates specific DNA binding. This idea is supported by the enhancement of Vmw65-DNA complex formation observed when Vmw65 has been denatured and renatured (Kristie and Sharp, 1990). Another possibility is that a high affinity GARAT binding region is shared between Vmw65 and another factor (for

example, Oct-1) that must be present to form a high affinity complex. The existence of a dimeric binding motif within the Type 2 TAATGARAT consensus was originally suggested by Sturm et al. (1989). This model is supported by the cooperative interaction observed with the formation of the Oct-1-Vmw65-TAATGARAT complex (Kristie and Sharp, 1990; Stern and Herr, 1991). Finally, it should be noted that the above two possibilities are not mutually exclusive.

The further elucidation of the relationship between Vmw65 structure and function will continue to be of importance in the study of transcription in general. The localization of domains required for interactions and associations with host transcription factors will allow the search for homologous cellular counterparts of Vmw65. In particular, the identification of the domain responsible for binding to the POU domain of Oct-1 (Stern and Herr, 1991) will facilitate the hunt for coactivating factors that function through their association with POU domain DNA binding proteins. There is good evidence for the existence of Oct-1 (and Oct-2) associated factors that may act to greatly diversify the function of these proteins as regulators of transcription (Pierani et al., 1990; Luo et al., 1992). The relationship of these factors to Vmw65 has yet to be determined. In addition, the knowledge of the Vmw65 functional domains will allow the identification of associated cellular transcription factors by

affinity chromatography. VCAF-1 is one cellular factor that has been identified solely by virtue of its interaction with Vmw65 (Xiao and Capone, 1989). Finally, further investigations into the structure and function of the AAD should reveal the mechanism by which this common activation motif stimulates gene expression.

EXAMINATION OF THE PROPERTIES OF THE VMW65 DEPENDENT COMPLEXES AND IDENTIFICATION OF A CELLULAR FACTOR INVOLVED IN COMPLEX ASSEMBLY

In order to fully understand the process by which Vmw65 redirects cellular gene expression early during an HSV infection, one must first identify the host (as well as the viral) factors involved. There are at least two different and separate sets of interactions required for the Vmw65 mediated transactivation to occur; one providing specificity though direct or indirect contacts with the TAATGARAT element, and the other involved in the actual stimulation of transcription through contacts with the pre-initiation complex.

The Vmw65 acidic activation domain (AAD) is thought to stimulate transcription by contacting components of the RNA pol II pre-initiation complex. These interactions have been the subject of intense investigation because of their importance in the understanding of basal and regulated transcription and the manner in which the two are linked. There is conflicting evidence as to whether the AAD indirectly contacts the transcription pre-initiation complex through transcriptional adaptors (called TAFs) (Berger *et al.*, 1990;

Kelleher *et al.*, 1990) or whether it directly binds to components of the complex; specifically, transcription factors TFIID (Stringer *et al.*, 1990; Ingles *et al.*, 1991), TFIIB (Lin and Green, 1991; Lin *et al.*, 1991) or both (as suggested by Sharp, 1991). The mechanism by which such interactions actually increase the levels of RNA pol II transcription is still very hypothetical. In addition, the Vmw65 AAD has been shown to alleviate histone H1 mediated repression of transcription on genes that are packaged in nucleosomes (Laybourn and Kadonaga, 1991). Both activation and derepression require a second set of interactions that permit Vmw65 to form a high affinity complex with the TAATGARAT enhancer element. The work described here is focused upon the assembly and composition of this multi-protein-DNA complex.

The inability of Vmw65 to strongly bind to TAATGARAT sequences *in vitro* initiated the search for cellular proteins that did. The ubiquitous octamer binding protein, Oct-1, was subsequently shown to form a high affinity complex with this recognition element and to be a component of the Vmw65 induced complex (O'Hare and Goding, 1988). The inability of purified Oct-1 and Vmw65, synthesized *in vitro*, to form a complex on the TAATGARAT element implicated the involvement of an additional factor(s) (Gerster and Roeder, 1988). This chapter describes several strategies that have been employed to identify novel components of VIC. Initially, detergents were

used to probe or dissect the multi-protein-DNA complex. Subsequently, HeLa cell extracts were fractionated to separate and/or deplete the individual activities required for complex assembly. Finally, the availability of recombinant Oct-1, along with the purified cell fractions has permitted the reconstitution of the Vmw65 dependent complex in the *in vitro* binding assay.

4.1 The Specific Effects of Detergents on the Vmw65 Dependent Complex

Some protein interactions (ie hydrophobic) are sensitive to low concentrations of detergents. This effect can be used to partially or completely disassemble a multi-protein complex into its constituent parts. Such experiments can be informative as to the composition of a complex and may also further the understanding of the pathway by which it assembles. This strategy has been successfully used in the dissection of the RNA pol I (Kato *et al.*, 1986), pol II (Hawley and Roeder, 1985, 1987) and pol III (Kovelman and Roeder, 1990) transcription pre-initiation complexes. The availability of the mobility shift assay allows a similar approach to be employed in the study of the Vmw65 induced complex.

Extensive studies were carried out to determine the

effect of various detergents on the Oct-1 and Vmw65 dependent complexes. Representative anionic, cationic and zwitterionic detergents were titrated into the standard mobility shift binding reaction and the qualitative and quantitative effects on complex formation were examined. Interestingly, the different detergents effected Oct-1 and Vmw65 complex formation in different and concentration dependent ways. In no case did the time of addition (ie before or after complex formation) appear to influence the overall effect of a particular detergent. The inability to produce a detergent stable complex suggests that the Vmw65 dependent complex formed in the binding reaction has a relatively short half life and is continuously associating and dissociating.

Mobility shift experiments in the presence of Sarkosyl revealed that this detergent has no effect on complex formation at concentrations less than 0.025 % (Fig. 4.1.1). When increased to 0.05 % Sarkosyl obstructs VIC without visibly effecting the Oct-1 complex. This result suggests that there are at least 2 steps to complex formation; step one, is resistant to 0.05 % Sarkosyl and involves the formation of the Oct-1 complex, step two, the assembly of VIC is sensitive to 0.05 % Sarkosyl. In higher concentrations (>0.1 %) Sarkosyl

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Fig. 4.1.1   Effects of Sarkosyl on the Oct-1 and Vmw65 induced complexes. The TAATGARAT probe was incubated with 8 µg of HeLa nuclear extract, alone or in the presence of 2 µg of Vmw65 (from HSV), as indicated. Reactions were supplemented with different concentrations of the detergent Sarkosyl (% v/v). Complexes were resolved on a native polyacrylamide gel.

interferes with the binding of Oct-1 to DNA and neither VIC nor the Oct-1 complex is observed. Under no conditions are novel, partially assembled complexes observed. The Sarkosyl concentrations used in this experiment are in the same range as those used by Roeder to disrupt the protein-protein interactions within the transcription pre-initiation complex (Hawley and Roeder, 1985).

The other anionic detergents tested (sodium dodecyl sulphate (SDS) and deoxycholate (DOC)) showed effects similar to Sarkosyl; they were capable of interfering with VIC formation at concentrations that had no effect on the Oct-1 complex (Fig. 4.1.2 and Table 4.1.1). Apparently, anionic detergents specifically interfere with a protein-protein interaction involved in the assembly of VIC. An additional effect was observed with DOC. This detergent enhanced the formation of a much smaller complex (Fig. 4.1.2, lanes g and f) that is faintly visible in the absence of the detergent. The identity of this DNA binding factor is not known.

Generally, non-ionic and zwitterionic detergents did not effect Oct-1 or VIC complex formation in concentrations below 0.5 % (w/v) (Fig. 4.1.2, Table 4.1.1). One exception was Triton X-100. 0.08-0.15 % Triton X-100 was observed to significantly and quantitatively increase the mobility of
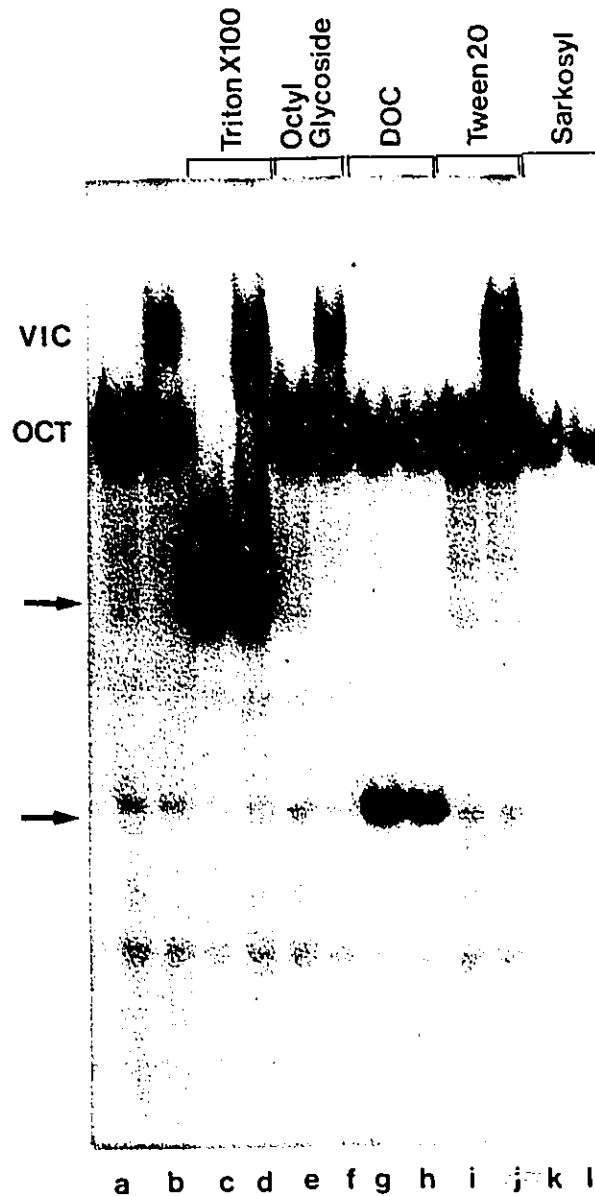
Fig. 4.1.2 Specific effect of different detergents on the formation of the Oct-1 and Vmw65 induced complexes. The labelled probe was incubated with 8 μg of nuclear extract (lanes a,c,e,g,i,k) or nuclear extract and 2 μg Vmw65 (from HSV) (lanes b,d,f,h,j,l). 1.25 % (v/v) of the indicated detergents were included in the appropriate lanes. Complexes were separated by native gel electrophoresis.

DETERGENT[a]	concent ration[b] (%w/v)	EFFECTS[c]
Sarkosyl	0.025 0.05 0.10	none obstructs VIC, no effect on Oct-1 obstructs Oct-1 and VIC
DOC	0.1	obstructs VIC
SDS	0.005 0.015 0.05	none obstructs VIC obstructs VIC and Oct-1
Triton X-100	0.025 0.05	none increases the mobility of Oct-1 complex
Tween	0.15	none
octyl-glycoside	0.15	none
CHAPS	0.15	none
TMAB	0.15	binds to probe

Table 4.1.1   Effects of Specific Detergents on Oct-1 and Vmw65 Induced Complex Formation.
[a]anionic detergents; Sarkosyl, deoxycholate (DOC), sodium dodecyl sulfate (SDS)
non-ionic detergents; Triton X-100, Tween 20, octylglucoside
zwitterionic detergent; CHAPS
cationic detergent; TMAB (tetradecyltrimethyl-ammoniumbromide)
[b]represents final concentration weight/volume in the binding reaction
[c]effect as observed in a mobility shift assay (see Fig. 4.1.2).

the Oct-1 complex but had little effect on VIC (lanes c and d). The mobility of a PA-POU (a protein A fusion to the POU domain of Oct-1, see section 4.3.7) TAATGARAT complex was not effected by Triton X-100 (at these concentrations). These observations suggest that the presence of this detergent induces a substantial conformational change in the full length Oct-1 protein without effecting the affinity or specificity of the Oct-1 POU domain for DNA or its ability to participate in the formation of VIC. Such a change in conformation could result in the increase in mobility on a non-denaturing gel. Alternatively, it is possible that a component of, what our lab and others have designated, the Oct-1-DNA complex can be selectively removed. Oct-1 associated factors have previously been detected by others (Pierani et al., 1990). It is also possible that the complex observed in the presence of Triton X-100 is not Oct-1 and instead is another protein which exhibits increased affinity under the conditions used.

The differential effects of specific detergents on multi-protein-DNA complex assembly can be used to investigate the composition and disposition of the structure in question. Further, the results may provide information regarding the pathway by which the complex assembles. These detergent studies have indicated that Vmw65 dependent complex assembly can be dissected into distinct steps in the presence of anionic detergents. In addition the Triton X-100 effect on

Oct-1 may be useful in future studies into the conformation and function of this protein. Overall this work has provided a starting point for a more stringent examination of the Vmw65 dependent complex.

4.2   Other Factors Involved in VIC Formation

Evidence for the existence of a VIC component(s), in addition to Oct-1 and Vmw65, was initially obtained through mobility shift experiments utilizing different combinations of nuclear extract and Vmw65 concentrations (Fig. 4.2.1). The amount of VIC formed is initially dose dependent upon the concentration of Vmw65 but then appears to reach a maximum level (lanes f-j). However, one observes a significant increase (20 to 30 fold) in the levels of VIC when the concentration of Vmw65 is kept constant and the amount of nuclear extract is increased (lanes k-o). Together these results suggest that a factor that is required for the assembly of VIC is present, in limiting concentrations, in the nuclear extract. The presence of significant quantities of the OCT complex in lanes n and o suggest that Oct-1 is not limiting in these reactions and thereby implies the existence of another component of VIC.

| a | b | c | d | e | | f | g | h | i | j | | k | l | m | n | o |

←VIC

←OCT

Nuclear Ext. (μg)	4	6	8	12	16		8	8	8	8	8		4	6	8	12	16
Vmw65 (μg)	–	–	–	–	–		.04	.1	.2	.4	.8		.1	.1	.1	.1	.1
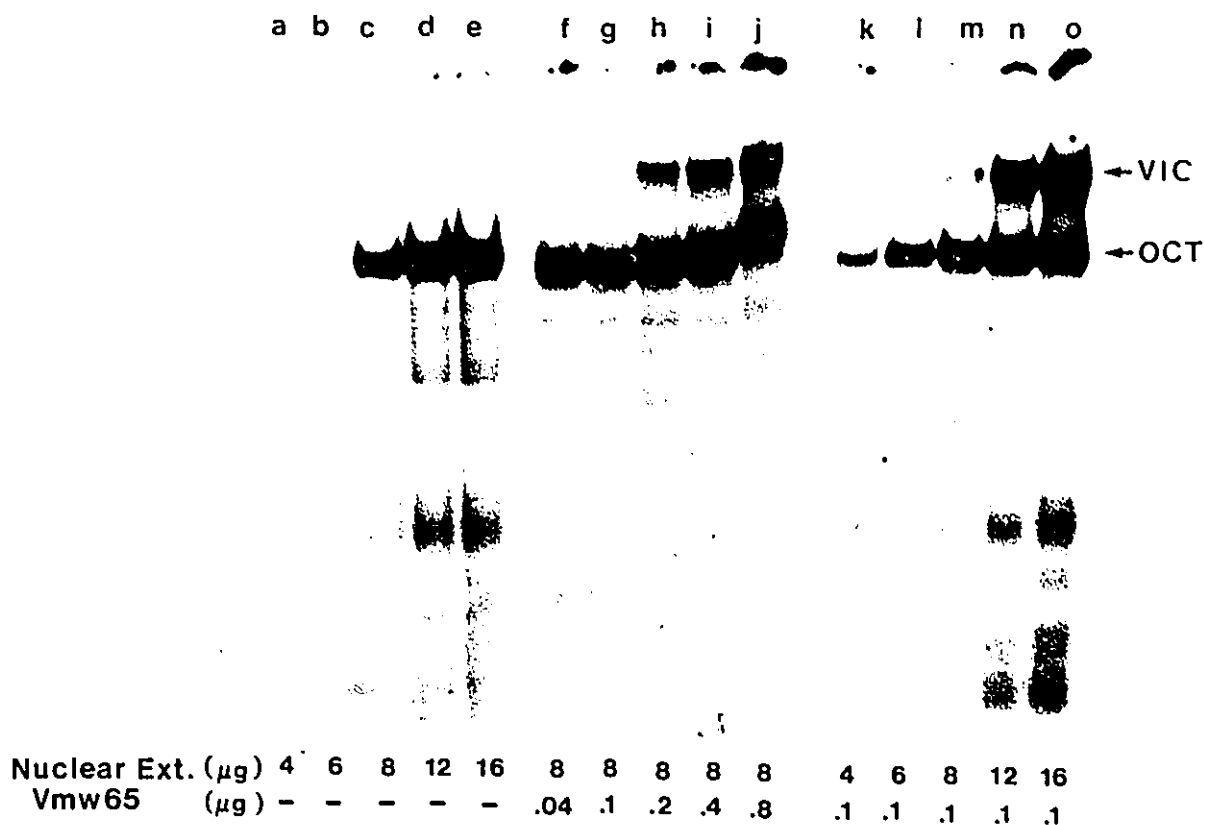
Fig. 4.2.1 The effects on VIC of various concentrations of Vmw65 and HeLa nuclear extract. Various concentrations of nuclear extract (lanes a-e) or nuclear extract and Vmw65 (lanes f-o) were incubated with the labelled wt probe as indicated. Complexes were separated by native gel electrophoresis. The positions of the Oct-1 (OCT) and VIC complexes are indicated.

The ability to produce and purify both Oct-1 and Vmw65 from *E. coli* has facilitated the search for other factors involved in the formation of the Vmw65 dependent complex. One such factor, VCAF-1, has been identified by virtue of its affinity for the protein Asa1 fusion protein (a truncated Vmw65 construct previously shown to be capable of driving VIC formation, see Fig. 3.4.5; Xiao and Capone, 1990). The depletion of VCAF-1 from a HeLa nuclear extract by affinity chromatography renders the extract incapable of supporting VIC formation. The addition of an active VCAF-1 fraction (from HeLa or rat liver cells) to bacterially produced Oct-1 and Vmw65 is sufficient to restore the complex (see Fig. 4.3.11). Other researchers have found activities that are similar, perhaps identical to VCAF-1. A VIC component, designated C1, has been identified in both HeLa and SF9 (insect) cell extracts (Kristie et al., 1989). Another factor, CFF, with an estimated molecular mass of 70 kDa, has been purified using chromatographic techniques from HeLa nuclear extracts (Katan et al., 1990). Finally, a factor, HCF, has been identified in HeLa cells by virtue of its affinity to the protein Asa1 construct (Stern and Herr, 1991). Each of these semi-purified factors have been shown to be required for the assembly of the Vmw65 dependent complex on a TAATGARAT element. None of the above factors, however, have been cloned and the possibility remains that more than one protein is required for VCAF-1 (C1,

CFF, HCF) activity.

4.3   Identification of a HeLa Cell Factor that Enhances VIC as Observed by Mobility Shift

Early experiments using varying amounts of Vmw65 and nuclear extract had suggested that some factor(s), other than Vmw65 and Oct-1, was limiting for Vmw65-dependent complex formation under our mobility shift assay conditions (Fig. 4.3.1). To investigate that possibility, the binding reaction was supplemented with various amounts of S100 post-nuclear extract. S100 extracts are capable of supporting pol III transcription (Wu, 1978) and may contain a wide variety of transcription factors. When S100 extract was incubated with probe in the absence of nuclear extract no specific DNA binding activity was detected indicating that the extract contains little or no Oct-1 binding activity (lane j). Incubation of S100 with Vmw65 also did not generate any protein-DNA complexes, indicating that the S100 extract does not contain a low affinity octamer binding protein that forms complexes only in the presence of Vmw65 (lane k). However, when a binding reaction containing nuclear extract and a virion extract (containing Vmw65) is supplemented with S100 extract the amount of VIC formed is significantly

a　b　c　d　e　f　g　h　i　j　k

VIC→

OCT→
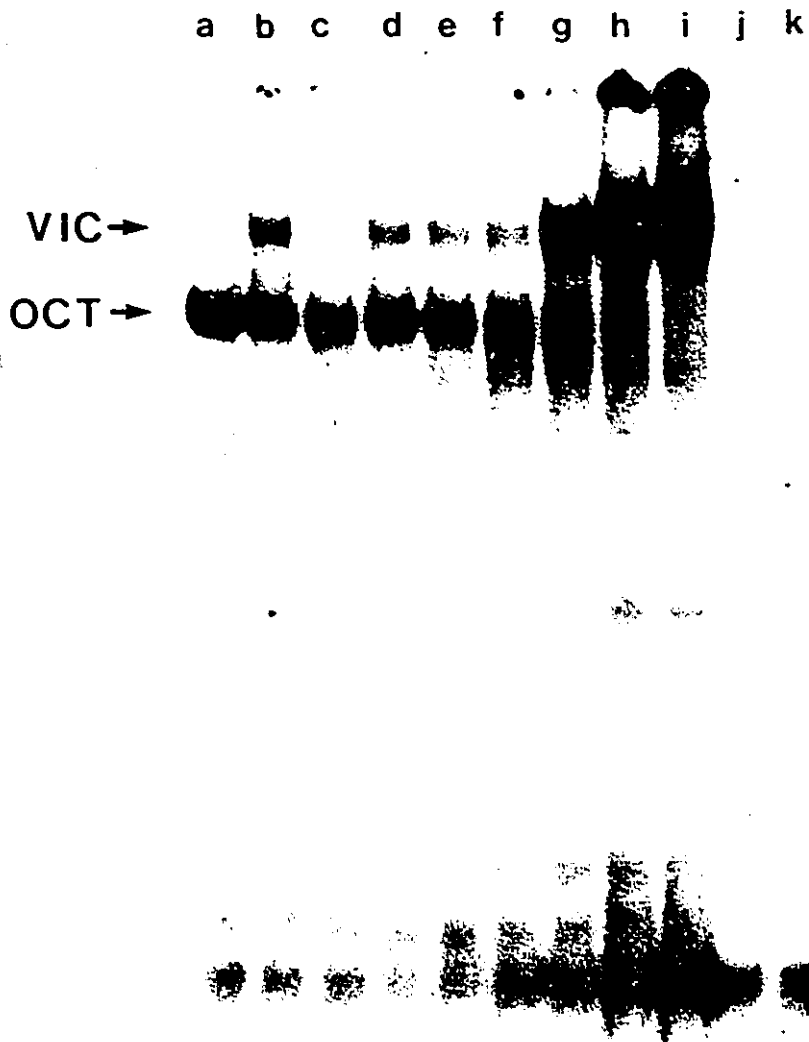
Fig.4.3.1　S100 stimulates Vmw65-dependent complex formation. HeLa nuclear extract (8 μg) was incubated with labelled probe in the absence (lane a) or presence (lane b) of 2 μg of HSV-1 virion extract (containing Vmw65). Lanes c to i were identical to lane b except that increasing amounts of HeLa cell S100 extract were added to the reactions at 0.05, 0.1, 0.15, 0.25, 0.5, 1.0 and 2.5 mg/ml respectively. Lanes j and k represents S100 extract (2.5 mg/ml) incubated with probe but without nuclear extract and in the absence (lane j) or presence (lane k) of 2 μg of Vmw65. All reactions were carried out in 15 μl volumes and normalized for protein concentrations (with BSA) and for buffer conditions. OCT; Oct-1 protein/DNA complex, VIC; Vmw65 dependent protein/DNA complex.

enhanced in a dose dependent manner (Fig. 4.3.1, lanes c-i).
Therefore a factor distinct from Oct-1 appears to be capable
of inducing complex formation. This factor has been designated
SF (stimulatory factor).

The stimulation of VIC formation by SF is dependent
upon assay conditions and especially the extract
concentrations used in the binding reactions. The SF effect is
most pronounced under conditions where sub-optimal
concentrations of nuclear extract or Vmw65 was used in the
binding reactions (Fig. 4.3.2). Under such circumstances, one
could observe up to a 20-fold increase in the amount of VIC
formed in reactions supplemented with S100 extract (compare
lanes b and c). Under conditions where the amount of nuclear
extract was increased in order to optimize VIC formation, then
the addition of S100 extract had much less of an effect on VIC
formation (compare lanes e and f with lanes b and c). This is
further demonstrated in lanes h and i which were identical to
lanes e and f, respectively, except that the reaction volume
was increased to 20 μl from 10 μl. Under these circumstances,
one could again see a significant effect on VIC formation
following addition of S100 extract (compare lane h with i).
The stimulatory effect was not observed when the amount of
nuclear extract used in these reactions was doubled (lanes k
and l). The enhancing effect is, therefore, highly dependent

NUCLEAR EXTRACT (µg)	8	8	8	16	16	16	16	16	16	32	32	32
Vmw65	-	+	+	-	+	+	-	+	+	-	+	+
S100 EXTRACT	-	-	+	-	-	+	-	-	+	-	-	+
VOLUME (µl)	10	10	10	10	10	10	20	20	20	20	20	20
	a	b	c	d	e	f	g	h	i	j	k	l
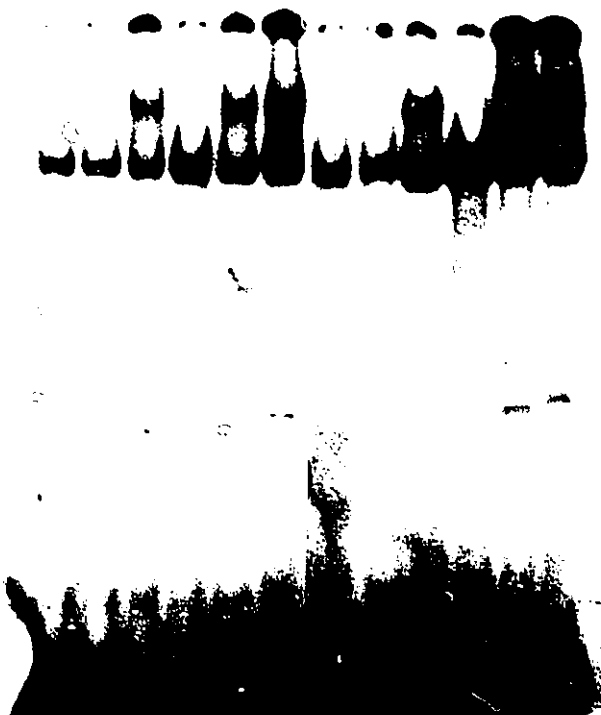
Fig. 4.3.2   Stimulation of VIC formation by S100 extract under different reaction conditions. The effect of adding S100 extract on VIC formation was tested under different conditions as indicated at the top of the figure. Vmw65 (from HSV) and S100 extract were added, where indicated, at 0.5 µg and 2.5 µg, respectively, per binding reaction. In lanes a-f, the reaction volume was 10 µl while in lanes g-l, the reaction volume was 20 µl. Protein concentrations and buffer conditions were normalized in all cases.

upon the concentration of nuclear extract in the binding reaction. These results suggest that S100 extract can compensate for a factor which, under appropriate experimental conditions, is present in limiting quantities in the nuclear extract.

As exhibited in the above experiment, assay conditions used in the binding reactions can have a dramatic effect on the amount of VIC complex formed. Therefore in all subsequent experiments assay conditions such as, protein concentration, reaction volumes, salt and other buffer components were carefully controlled and normalized.

4.3.1 Specificity of the enhancing effect for VIC assembly

The factor responsible for stimulating VIC could act in a general fashion by increasing the affinity of protein-protein and/or protein-DNA interactions non-specifically (in a manner similar to that observed with BSA and albumin (Kozmik et al., 1990; Zhang et al., 1992)). Alternatively, SF could enhance the interactions of a specific component of VIC. The addition of S100 extract to nuclear extract in the absence of Vmw65 has no effect on the amount of Oct-1 complex formed (Fig. 4.3.4). In similar experiments the S100 extract has no apparent effect on the independent binding of Vmw65 to TAATGARAT in the absence of nuclear extract or on the

cooperative binding of Vmw65 and Oct-1 to DNA. This latter observation distinguishes SF from VCAF-1. The binding of proteins supplied by a Fm3a (mouse mammary tumour cell) nuclear extract to an AP-1 consensus sequence were also unaffected by the presence of this extract (data not shown). The results of these experiments suggest that SF is not a non-specific enhancer of protein-protein interactions. Therefore one must conclude that the enhancing effect is in some way specific for the assembly Vmw65 induced complex.

4.3.2 The physical properties of SF

To further delineate the mechanism behind SF activity a more pure form of the factor was required. The process of purification could also greatly assist in the characterization and eventual identification of the factor. To further characterize SF, S100 extract was sequentially fractionated by ultrafiltration using Centricon microconcentrators equipped with various molecular weight cut-off filters. The enhancing activity was followed through each step with the use of the mobility shift assay. SF activity was eventually recovered in the filtrate following centrifugation with the Centricon 3 indicating the factor is less than 3000 Da in size (Fig.4.3.3A, lanes b-f). This experiment also demonstrates that SF enhances complex formation in a dose dependent manner
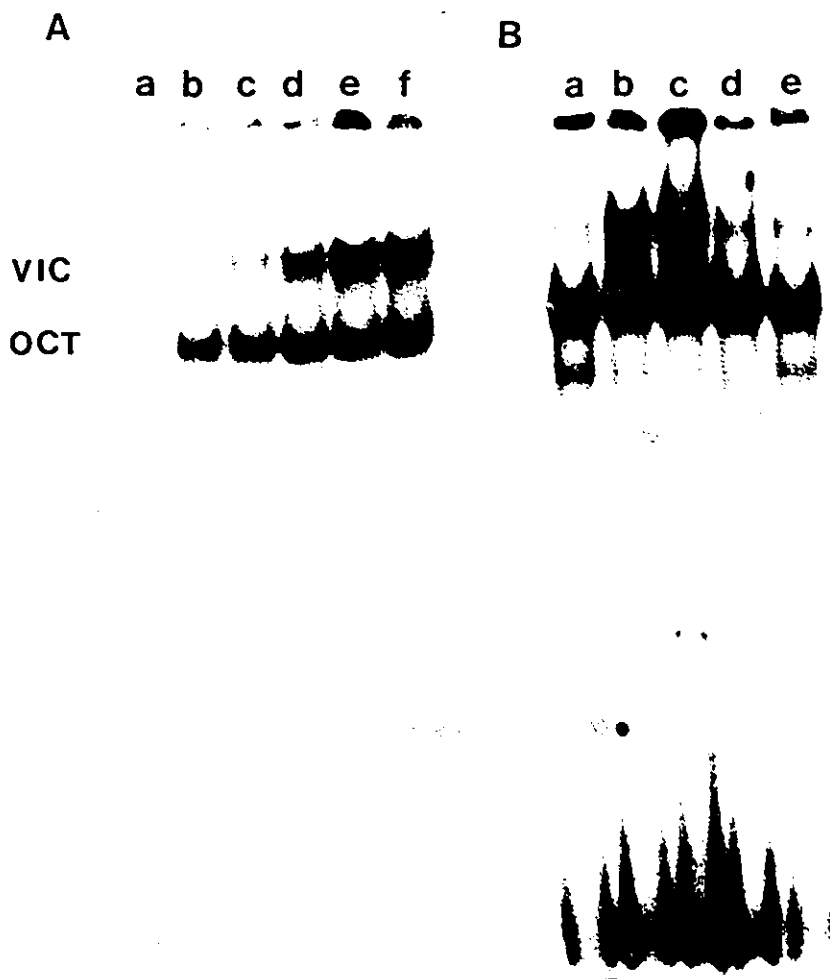
Fig 4.3.3   Size estimation of the stimulatory factor. A) SF activity was present in the filtrate following ultrafiltration using a 3000 Da cut-off filter. HeLa nuclear extract (8 µg) was incubated with Vmw65 (2 µg) in a final volume of 20 µl in the absence (lane b) or the presence of 3, 6, 9 or 12 µl of the Centricon C-3 filtrate (lanes c-f, respectively). In lane a, the C3 filtrate (6 µl) was incubated with Vmw65 in the absence of nuclear extract.   **B)** The C3 filtrate was subjected to size exclusion chromatography and 12 µl of the excluded fraction from each column was added to a binding reaction as in lane b. No addition (lane a), excluded fraction from Sephadex G10 (lane b), G15 (lane c), G25 (lane d) and G50 (lane e), respectively.

(as does the activity in the crude S100 extract). In order to better estimate the molecular weight, the C3 filtrate was subjected to size exclusion chromatography with Sephadex beads having different exclusion limits. 100 μl of C3 filtrate was loaded onto 1 ml Sephadex columns and the excluded volume was collected and tested for activity in each case (Fig. 4.3.3B). SF activity was excluded from the G10 and G15 gels (lanes b and c), a characteristic typical of molecules larger than 700 and 1500 Da, respectively. This result is important because it sets a lower limit on the size of SF (1500 Da) and removes any possibility that the enhancing activity is a buffer effect. However, the activity was retained in the G25 (fractionation range 100-5000 Da) and G50 (fractionation range 500-30 000) columns (lanes d and e,). This result puts an upper limit on molecular weight (5000 Da) that is consistent with the ultrafiltration data. Together these data suggest that the molecular weight of SF lies between 1500-3000 Da.

To further purify SF (see Fig. 4.3.4), the active C3 fraction was separated by anion exchange chromatography on a 2 ml DEAE Sephacel column. The activity was step eluted with 1.25-1.5 M KCl and desalted by gel filtration chromatography on a Sephadex G15 column from which it was excluded. Phospholipids, peptides and other hydrophobic molecules were separated from the active fraction by Bligh Dyer (Bligh and

```
HeLa Cells
    |                    cell
    |                 fractionation
  S100
    |
    |
Centricon 30
    |filtrate
    |                    ultra-
Centricon 10            filtration
    |filtrate
    |
Centricon 3
    |filtrate
    |                      ion
DEAE Sephacel           exchange
    |1.25 M KCl
    |                      gel
Sephadex G15          filtration
    | flow
    |through
                         organic
Bligh Dyer Extraction   extraction
    |aqueous
    |
   SF
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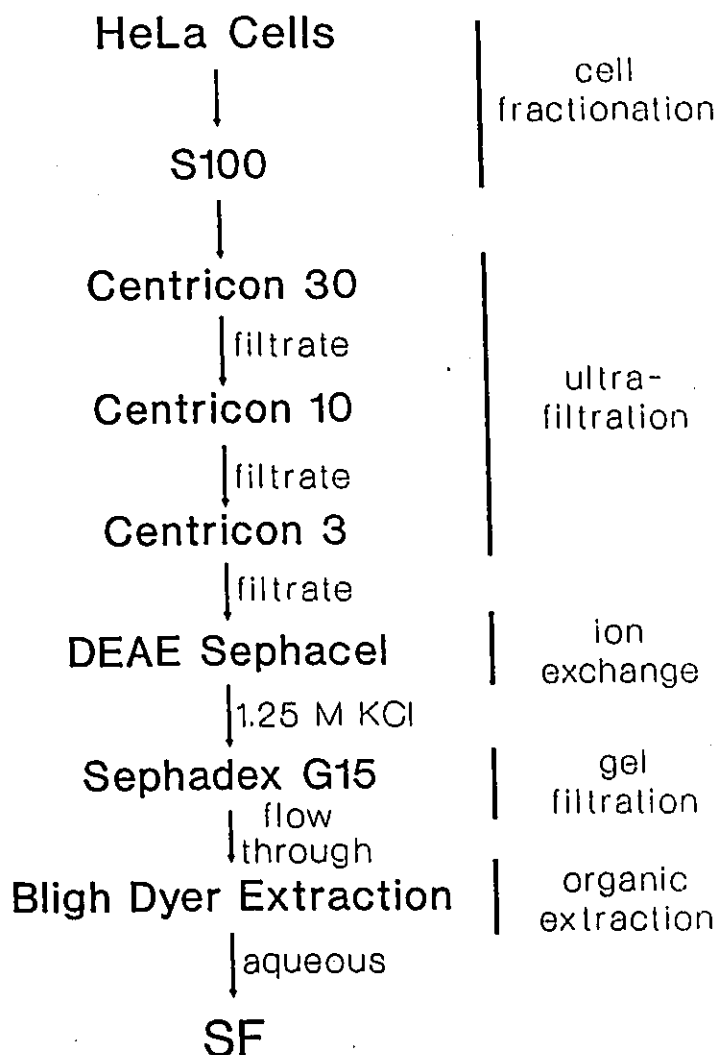
Fig. 4.3.4   Procedure for the purification of SF Activity from HeLa cells. S100 extracts, prepared from HeLa cells grown in suspension culture, were fractionated using a series of Centricon ultrafiltration devices of decreasing molecular weight cut-off. The filtrate from the C3 filter (MW cut-off 3000 Da) was loaded onto a DEAE Sephacel column. Enhancing activity was eluted with 1.25 M Kcl. The fraction containing SF activity was desalted by gel filtration on a Sephadex G15 column. This step would remove all components of the fraction with MW less than approximately 1500 Da. Remaining hydrophobic contaminants were then removed by Bligh Dyer (methanol-chloroform) extraction. SF activity was monitored through each step of the purification procedure using the mobility shift assay.
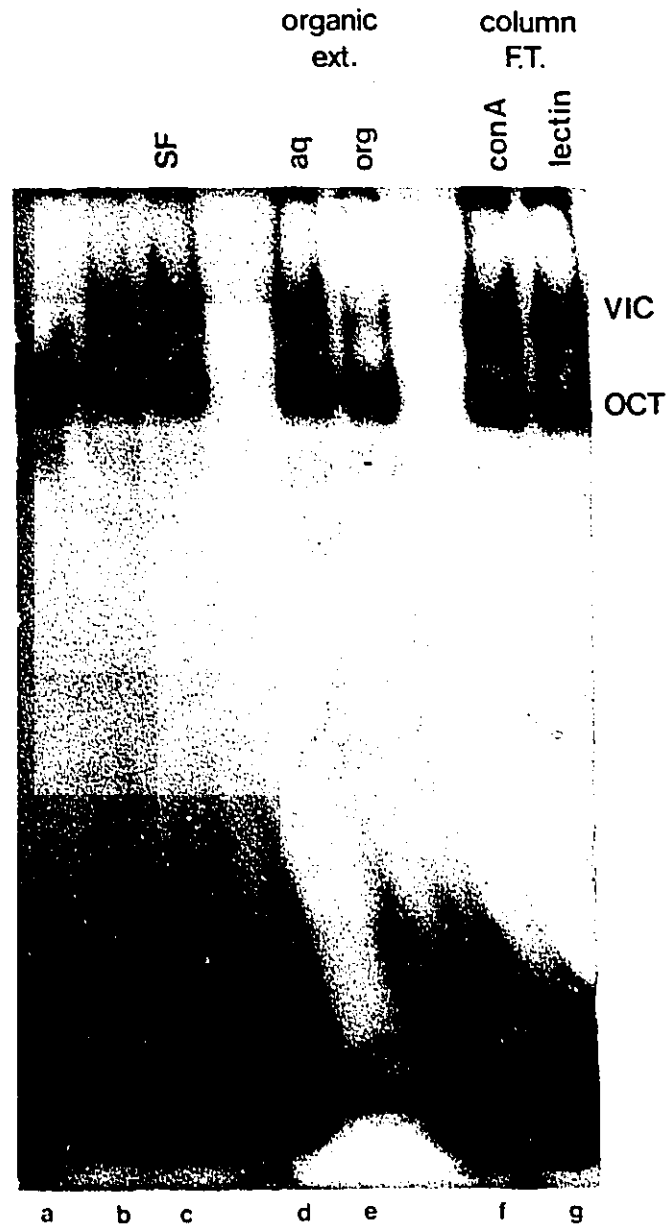
Fig. 4.3.5 Monitoring the purification of SF. The wt mobility shift probe was incubated with HeLa nuclear extract alone (lane a), or in the presence of 2 μg A65 (lanes b-g). Binding reactions were supplemented with SF fractions from various stages of the purification procedure (see figure 4.3.4); 4 μl S100 Centricon 3 filtrate (lane c), 4 μl of the aqueous or organic soluble fractions from a Bligh Dyer extraction (lanes d and e, respectively), or 4 μl of the flow through from the separation of the most pure SF fractions on Con A and lentil lectin columns (lane f and g, respectively).

Dyer, 1989) organic extraction (methanol/chloroform). Both aqueous and organic phases were tested by mobility shift. SF activity was determined to partition into the aqueous phase (Fig. 4.3.5). The remainder of the experiments described in this section utilized SF that had been purified to this stage.

Attempts to further purify this factor were inconclusive. SF was not retained on an Asal affinity column suggesting that, under the conditions tested, it does not strongly interact with Vmw65. TLC separation and analysis of the most-pure SF fraction indicated the possible presence of a carbohydrate containing molecule. Carbohydrates were visualized by spraying the silica plate with sulphuric acid and methanol (1:3 v/v) and then incubating it at 110 °C for 10 minutes (Robyt and White, 1987). However, SF activity had no affinity for ConA or lentil lectin columns (Fig. 4.3.5).

4.3.3 Characterization of SF through treatment with digestive enzymes

In an attempt to identify the composition of the molecule responsible for the enhancing activity, an active SF fraction was treated with various degradative enzymes. Any effect on activity was monitored with the mobility shift assay. The results indicate that SF activity is not affected by heat treatment (100°C for 15 min) nor by extensive treatment with degradative enzymes including trypsin,

proteinase K, RNAse A, DNAse I, or phospholipase A_2 (Fig. 4.3.6, lanes d-j). Thus, SF does not appear to contain essential nucleic acid and if it is proteinaceous, it is highly resistant to proteases and heat denaturation.

The presence of a carbohydrate containing substance was indicated by TLC analysis of the most purified SF fraction. To investigate the possibility that SF has a carbohydrate component the C3 filtrate was treated with a series of exoglycosidases and the affects on enhancing activity were assayed by mobility shift. Pretreatment with β-glucuronidase had no effect on the ability of SF to enhance complex stability but the resulting complex was significantly decreased in its mobility (Fig. 4.3.7, lane d). An identical pretreatment of buffer D had no affect when added to the binding reaction in the presence or absence of the enhancing activity (lanes c and e, respectively). Similar treatment with a number of other glycosidases (Table 4.3.1) had no apparent affect on the ability of SF to enhance complex formation or on complex mobility. It is important to note that the glycosidase enzymes are not pure preparations. ß-glucuronidase commonly contains contaminating activities, the most typical being arylsulfatase. Arylsulfatase was tested and had no effect on SF activity. Still, there is a possibility that the shift in
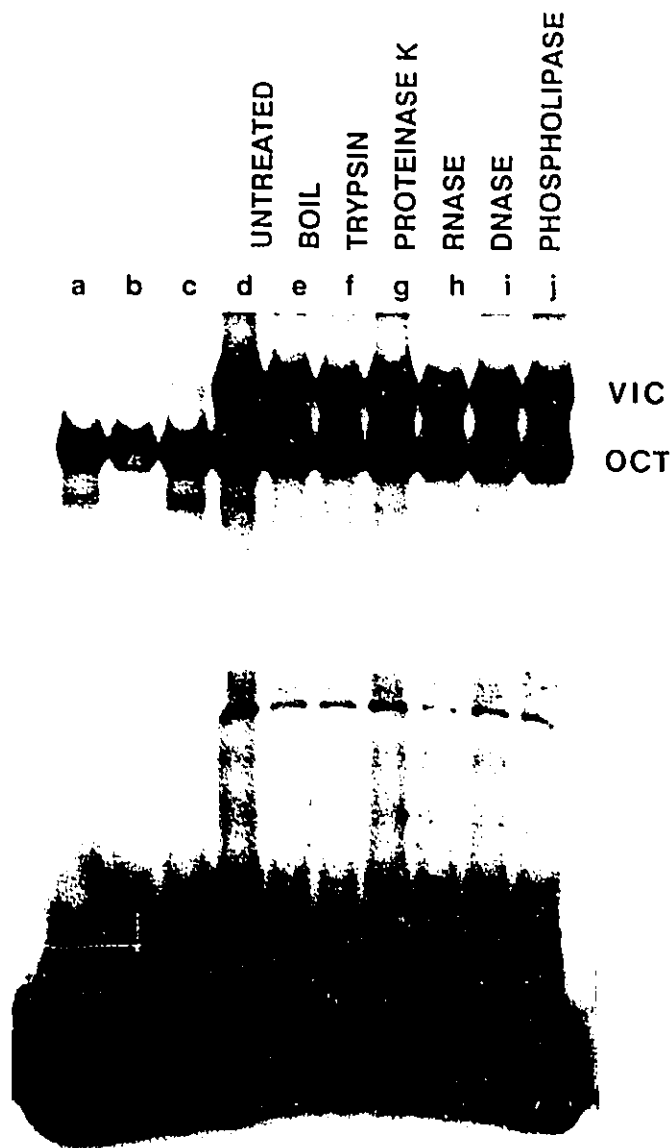
**Fig. 4.3.6   The stimulatory activity is resistant to
heat inactivation and digestion with degradative enzymes.**
Vmw65 (0.5 μg of viral extract) was incubated with 8 μg of
nuclear extract in the absence (lane c) or presence of 4 μl of
C3 filtrate which was untreated (lane d), heat treated for 15
minutes at 100°C (lane e), or treated for 2 hours at 37°C with
trypsin (50 μg/ml), proteinase K (50 μg/ml), RNAse A (10
μg/ml), DNAse I (10 μg/ml) or phospholipase A₂ (100 μg/ml)
followed by incubation at 100°C for 15 minutes, respectively
as indicated at the top of the figure.   Lanes a and b
represent nuclear extract incubated without Vmw65 in the
absence (lane a) or presence (lane b) of 4 μl of the C3
filtrate.
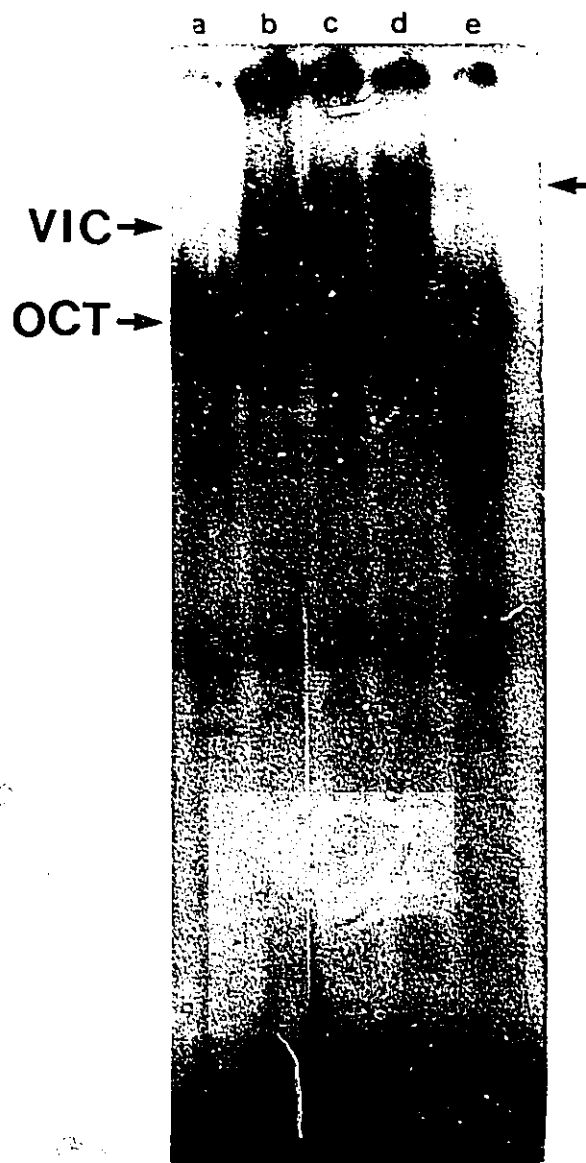
Fig. 4.3.7   Pretreatment of SF with β-glucuronidase results in a complex of decreased mobility. HeLa nuclear extract (8 μg) and Vmw65 (2 μg) were incubated in the absence (lane a) or in the presence of SF which was untreated (lanes b and c) or pretreated with 700 U/ml β-glucuronidase (lane d). Lanes c and e contain buffer D that has been pretreated with β-glucuronidase in an identical manner. In all cases β-glucuronidase treatment was followed by incubation at 100°C for 15 min.

EXOGLYCOSIDASE[a,b]	SPECIFICITY	EFFECT[c]
ß-N-Acetyl-D-glucosaminidase	GlcNAcß1-↓-X	−
ß-Amylase	Glcα1-4Glcα1-↓-X	−
Amyloglycosidase	Glcα1-↓-4Glcα1-X or Glcα1-↓-6Glcα1-X	−
ß-Fructosidase	X1-↓-2ßFru	−
α-Galactosidase	Galα1-↓-X	−
ß-Galactosidase	Galß1-↓-X	−
α-Glucosidase	Glcα1-↓-X	−
ß-Glucosidase	Hexß1-↓-X	−
ß-Glucuronidase	GlcAß1-↓-X	+
Neuraminidase	NANAα2-↓-X or NGNAα2-↓-X	−

Table 4.3.1   A list of the glycosidases used to treat SF.
[a]All enzymes were purchased from Boehringer Mannheim.
[b]Each glycosidase was used according to manufacturer's instructions.
[c]SF was pretreated with each of the indicated exoglycosidases and activity was tested in the mobility shift assay. (−) indicates the pretreatment had no effect on the ability of SF to enhance the Vmw65 dependent complex. (+) SF pretreated with ß-glucuronidase and added to the binding reaction resulted in a complex that exhibited a significant decrease in mobility.

mobility, observed upon the treatment of SF, is in fact due to some other component of the enzyme extracts. The effect of β-glucuronidase digestion of SF on the mobility of VIC suggests that SF is a component of this complex. The fact that this treatment resulted in a significant decrease in complex mobility is somewhat surprising. It is possible that the removal of the negatively charged glucuronic acid residues result in a decrease in complex mobility in a native gel. Another explanation is that the β-glucuronidase digested form of SF induces a conformational change and/or permits the association of a much larger factor(s) resulting in a complex of decreased mobility.

Glucuronic acid is utilized in the detoxification of numerous cellular molecules through the formation of glucuronides. The conjugation of this carbohydrate moiety to various xenobiotic and endogenous substances facilitates the export of the polar metabolites from the cell (Kasper and Henton, 1980). Glucuronidating activity is widely distributed in various tissues of many different species. The highest levels of activity are normally found in the liver (Kasper and Henton, 1980). It is not known if HeLa cells contain this glucuronidating activity. The range of substrates include various phenols (steroids), alcohols (tertiary amyl), carboxylic acids (benzoic and bilirubin) and amines (aniline). It follows that there can exist a huge number of substances

containing a glucuronic acid residue within a cell (Kasper and Henton, 1980). The effect of ß-glucuronidase digestion on the enhancing activity suggests that the molecule responsible for the enhancing activity may be a glucuronide.

4.3.4 A VIC enhancing activity is also present in HeLa nuclear extracts

The results shown in Fig. 4.3.8 suggest that SF is present in nuclear extracts as one might expect if it plays a role in Vmw65-dependent complex formation *in vivo*. The observation that SF is resistant to protease digestion and heat treatment allowed this to be determined. Nuclear extract was extensively digested with proteinase K and heated to 100°C for 15 minutes. After removal of insoluble material by centrifugation, the supernatant was assayed for VIC enhancing activity. Protease/heat treatment removes all Oct-1 protein-DNA binding activity in the nuclear extract (Fig. 4.3.8, lanes e) and no complexes are observed when Vmw65 is added to the treated extract (lane f). However, the treated extract is capable of stimulating VIC formation when added to untreated nuclear extract and Vmw65 (compare lanes b and d). Supplementing nuclear extract with treated extract in the absence of Vmw65 has no effect on the amount of Oct-1 complex formed (compare lanes a and c) indicating that the enhancing

a b c d e f

VIC

OCT
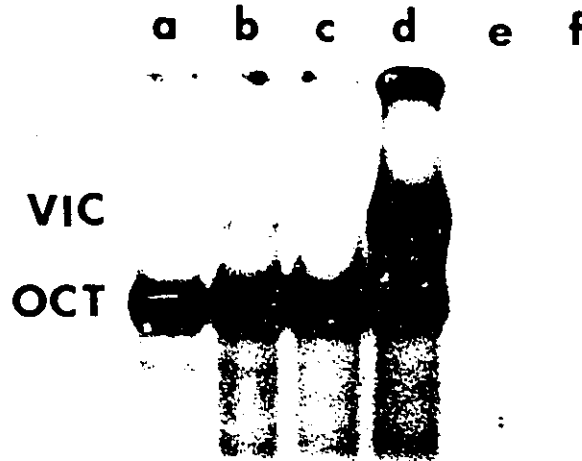
Fig. 4.3.8   SF activity is present in nuclear extracts. HeLa nuclear extract was treated with 50 μg/ml proteinase K for 2 hr at 37°C followed by heating at 100°C for 15 minutes. 2 μg of the clarified extract (equivalent to 16 μg of nuclear extract before treatment) was added to 8 μg of untreated nuclear extract in the absence (lane c) or presence (lane d) of 0.5 μg of Vmw65 (from viral extract). Lanes a and b are equivalent to lanes c and d respectively, except that the reactions were not supplemented with treated nuclear extract. In lanes e and f, treated nuclear extract (8 μg) was incubated in the absence or presence of Vmw65, respectively. All reactions were identical with respect to protein concentration and buffer composition.

effect is specific for VIC. This suggests that the stimulatory factor detected in post-nuclear extracts is also present in the nucleus.

4.3.5   SF acts to stabilize VIC

There are at least two ways by which the addition of SF to a binding reaction could enhance the resulting Vmw65 dependent complex observed by mobility shift. This factor could act by recruiting complex components and thereby stimulating the assembly of VIC or SF could stabilize the multi-protein-DNA complex after it has formed. To distinguish between these two possibilities the stability of VIC was compared in binding reactions that have been supplemented or not supplemented with the active SF fraction. Binding reactions in the presence or absence of purified SF were diluted four fold with buffer at set times during the incubation. The protein-DNA complexes from each time point were separated on a native gel and individually quantitated. Under the conditions of increased volume no detectable VIC is observed in the presence or absence of SF activity (see Fig. 4.3.2). Therefore preformed complex stability and not complex formation could be measured. When the binding reaction is supplemented with purified SF, the Vmw65 induced complex is stable for at least 30 minutes (Fig. 4.3.9, lanes f-i),
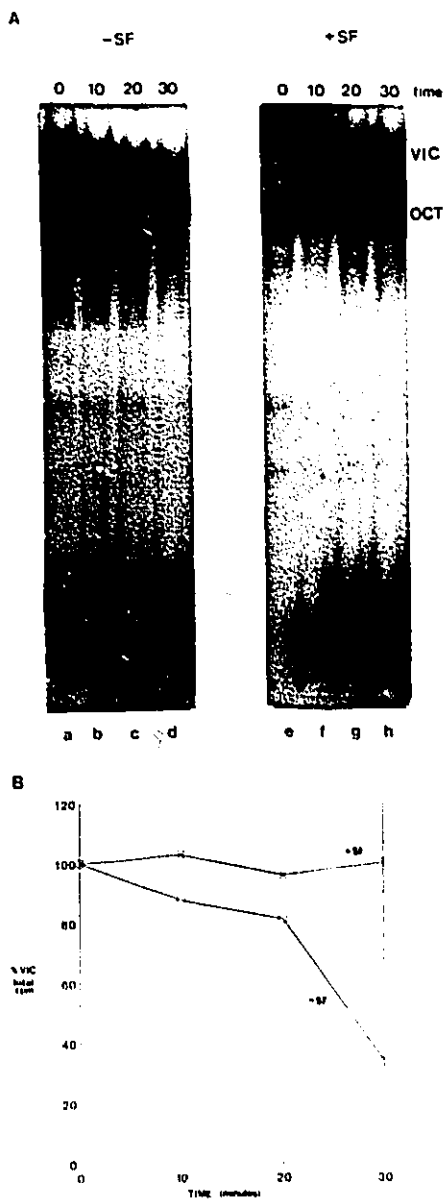
Fig. 4.3.9 A) SF activity stabilizes the Vmw65 dependent complex. Binding reactions were set up containing nuclear extract (8 μg) alone (lane a), in the presence of 0.5 μg Vmw65 (lanes b-e), or in the presence of 0.5 μg Vmw65 and 4 μl of purified SF (lanes f-i) in a total volume of 15 μl. Complexes were allowed to form for 5 minutes at 30°C after which reactions e and i were diluted with 45 μl of binding buffer. Reactions d and h were diluted 10 minutes later; c and g, 20 minutes later ; and a, b and f 30 minutes later (as indicated on the figure). **B)** The results of the experiment described above were averaged with data from three similar experiments and expressed in the form of a graph.

however, under identical conditions in the absence of the SF
fraction the VIC has almost completely dissociated by the 30
minute time point (lanes b-e). This result indicates that SF
is a limiting factor that acts in some manner to maintain VIC
in its assembled form. Because SF is present in the nuclear
extracts these data give no indication as to whether or not
this factor is essential for complex stability.

4.3.6   SF appears to be essential for VIC stability

To more carefully investigate the role of SF in the
binding reaction and to address the question as to whether
this factor is essential, an attempt was made to remove the
enhancing activity from a HeLa nuclear extract. This was
accomplished though extended dialysis with multiple changes of
the dialysis buffer. SF, being a molecule of 1500-3000 Da,
would presumably diffuse away from the larger components of
the extract which would become depleted of SF activity. After
the standard dialysis for 5 hours the nuclear extract is fully
capable of forming VIC in the presence of 0.5 to 2.0 μg of
Vmw65 (Fig. 4.3.10, lane b-d, respectively). However, the
extended dialysis (48 hours) of the same extract renders it
deficient for the ability to form VIC under identical
conditions (lanes e-g). Significantly, over-
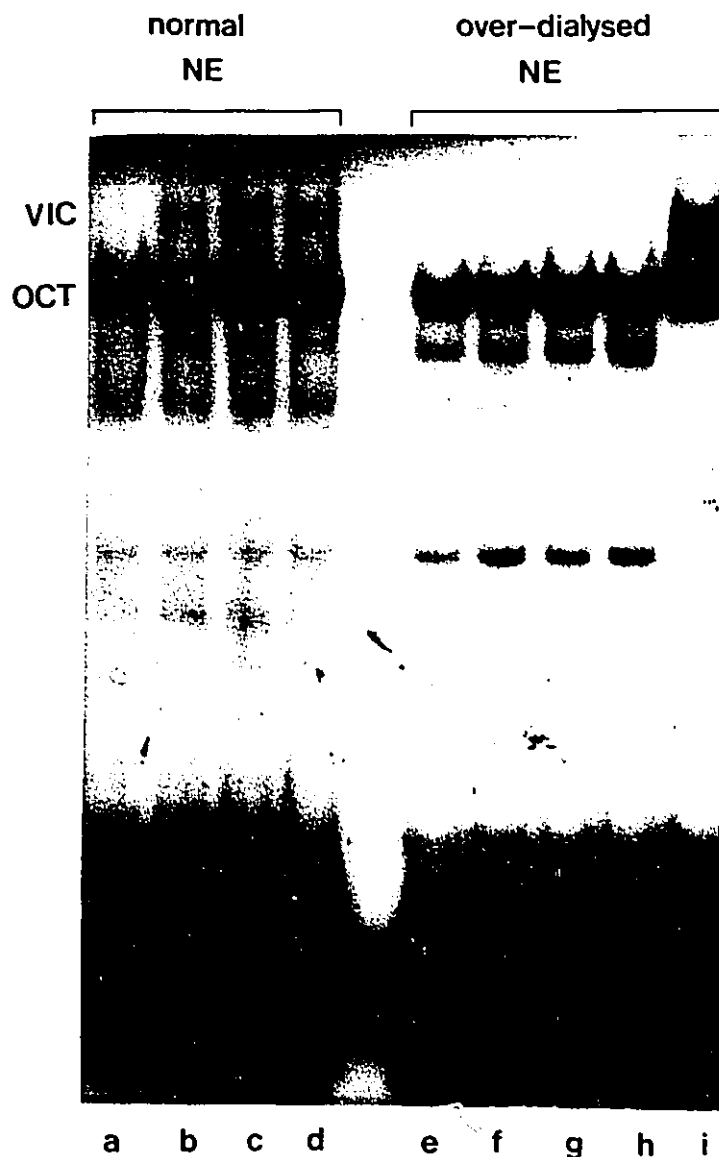
Fig. 4.3.10   SF can be depleted from a HeLa nuclear extract. HeLa nuclear extracts were dialysed in the normal manner for 5 hours or were over-dialysed for 48 hours in buffer D as indicated at the top of the figure. 8 μg of the appropriate nuclear extract was incubated with the mobility shift probe alone (lanes a,e) or with 0.5 μg (lane b,f,i), 1.0 μg (lanes c,g) or 2.0 μg (lanes d,h) of Vmw65 prepared from HSV virions. Lane i was also supplemented with 4 μl of the most purified SF fraction. Complexes were separated on a native polyacrylamide gel. As indicated, the over-dialysed extract was very inefficient at supporting VIC formation but this defect could be rescued by the addition of SF.

dialysis has no effect on the ability of the extract to form the Oct-1-DNA complex indicating that the treatment has no detrimental effect on Oct-1 (compare lane a and e). Finally, if the observed defect in the over-dialysed extract is due to a lack of SF then this should be remedied by the addition of the purified factor. As shown (lane i), the most-pure SF fraction is capable of restoring the capability of forming VIC to the depleted extract. This result suggests that the over-dialysed nuclear extract is depleted of SF, that with the exception of SF the extract is fully capable of forming VIC and, most significantly, that SF is essential for VIC formation under these conditions.

A more refined way of determining the necessity of SF in the Vmw65 dependent complex is to reassemble the complex using purified components. Oct-1 and Vmw65 were produced as protein A fusions, PA-POU and A65, respectively and VCAF-1 activity was supplied in the form of a rat liver cell nuclear fraction eluted from a Q column with 0.18 M KCl. The addition of SF to a binding reaction containing PA-POU, A65 and VCAF-1 resulted in a 30 fold increase in the amount of VIC observed on a mobility shift gel (Fig. 4.3.11, compare lanes f and i). Thus SF has the ability to enhance VIC assembled from purified and semi-purified components. In the absence of VCAF-1 (lane j) or in the presence of heat inactivated VCAF-1 (not shown) no VIC formation is observed confirming that VCAF-1 is
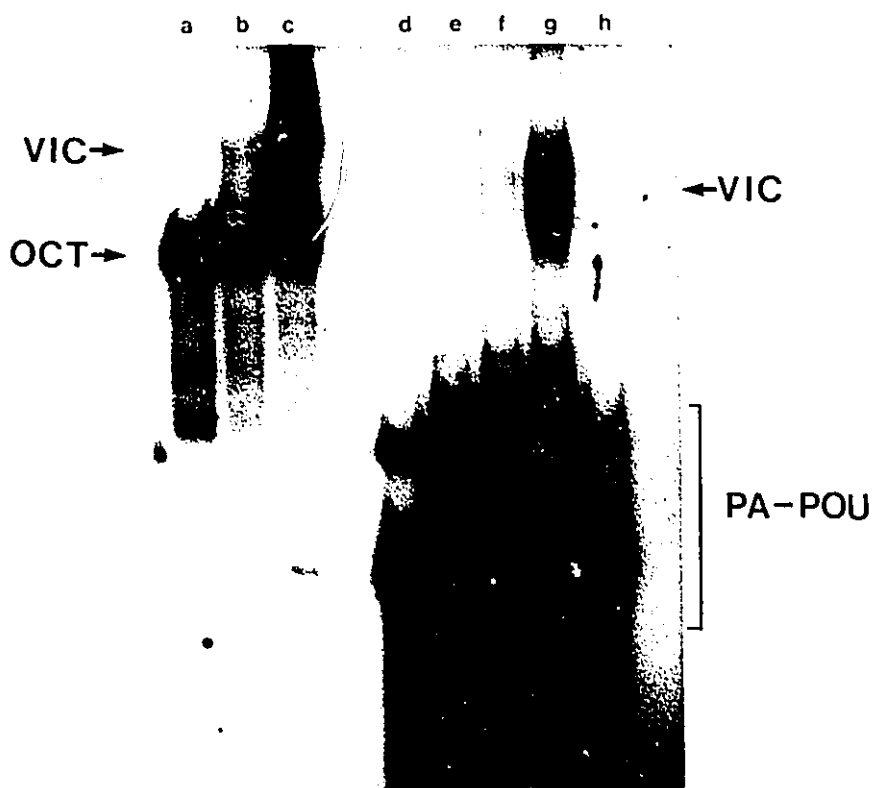
Fig. 4.3.11   SF enhances the reconstituted Vmw65 dependent complex. PA-POU (0.1 μg) was incubated alone (lane d), with A65 (0.5 μg) (lane e) or with A65 and VCAF-1 (lane f). Lanes g and h are similar to lane f but have been supplemented 4 μl of SF. In lane h the VCAF-1 fraction has been incubated at 80°C for 5 minutes prior to its addition to the biding reaction. As controls, nuclear extract (8 μg) was incubated alone (lane a), with A65 (lane b) are with A65 and 4 μl SF (lane c). PA-POU alone forms 2 distinct complexes with the probe. The lower mobility complex may represent a PA-POU dimer (Pomerantz et al., 1992).

essential for complex formation and that SF and VCAF-1 are distinctly different factors. A relatively small but detectable amount of VIC is observed in the absence of SF (lane f). This may indicate that low levels of SF activity are present in the crude VCAF-1 fraction. This is a credible explanation because these two factors have similar elution profiles. Of course it is also possible that the complex can inefficiently assemble in the absence of SF. A more highly purified form of VCAF-1 is required before either of these possibilities can be tested.

4.3.7 SF has no detectable effect in a DNAse I protection assay

The addition of SF stabilizes the Vmw65 dependent complex with no detectable effect on mobility in a native gel. However, the information obtained from a mobility shift experiment can be limited by the inability of this procedure to resolve different protein-DNA complexes that have similar migration characteristics. The DNAse I protection assay (Galas and Schmitz, 1978) was employed to more closely monitor the potential effects of SF on VIC. Specifically, protection assays are useful to examine the positioning as well as the stability of multi-protein complexes on DNA.

Complexes were pre-formed on DNA templates containing an identical TAATGARAT element as was used in the mobility

shift experiments (the promoter proximal IE0 sequence). Assays were attempted with HeLa nuclear extract but the non-specific DNA-binding activity in these crude extracts interfered with the specific protection pattern. Therefore purified, bacterially produced proteins were utilized to assemble the complex. The end-labelled DNA template was incubated with an increasing concentration of the purified A-POU fusion protein (Fig. 4.3.12, lanes b and c). The observed DNAse I protection pattern for A-POU alone extends both upstream and downstream of the octamer consensus, ATGCTAATGA. It is possible that the protein A tail extends the region of protection although a similar result was obtained with the GST-POU construct (data not shown). As a consequence, the sequential addition of A65 (lane f) and VCAF-1 (lane g) did not effect the protection pattern observed. The assembly of VIC was most probably masked by the extended A-POU footprint. In some experiments the addition of SF appeared to extend the footprint downstream (lanes h to j) although this effect was not always observed and may have been a result of increased complex stability. No change in the protection pattern was observed when the SF fraction was treated with ß-glucuronidase.

Fig. 4.3.12   The DNAse I protection pattern of VIC.
A 129 bp DNA fragment, containing the same IE0 TAATGARAT
element that was utilized in the mobility shift assay, was
labelled on a single strand. 50 000 cpm of probe was incubated
alone (lanes a,d and k) or with the indicated factors before
treatment with DNAseI. The DNA fragments were resolved on a
10% polyacrylamide gel. In order to map the DNAseI protection
pattern to a specific sequence the DNA template was sequenced
by the Maxam-Gilbert method. The G and G/A specific reactions
as well as the ATGCTAATGARAT sequence are indicated on the
figure.

DNAse I protection results similar to those described above have been observed by Kristie and Roizman (1987) with an IE27 TAATGARAT element and cellular extracts. The protection pattern was large (20 bp), extending both upstream and downstream of the TAATGARAT sequence, and no difference was observed when proteins were supplied by infected or mock-infected extracts. Kristie *et al.* (1989) reported that DNAse I protection extended at least 4 bp upstream of the same IEO octamer sequence used in the assays described here. However, in these experiments an extended footprint was observed in the presence of Vmw65. Preston *et al.* (1988) observed a tight 13 bp VIC footprint. It should be noted that all of the above experiments made use of full length Oct-1 and wild type Vmw65 whereas the assays described in this research utilized truncated Oct-1 and Vmw65 produced in *E. coli*.

In summary, the DNAse I protection results proved inconclusive in regard to further elucidating the nature of the Vmw65 dependent complex. It is apparent from the above collection of data that, due to the sensitivity of this assay, variations in conditions, components or techniques can lead to wildly different results. In this case it may be beneficial to combine the mobility shift and protection assays in order to separate the Oct-1, Oct-1-Vmw65 and VIC complexes before the DNAse treatment.

4.3.8   Speculation on the role of SF

SF is a small molecule present in both nuclear and post-nuclear HeLa extracts that is resistant to heat treatment as well as extensive digestion with proteases and nucleases. These characteristics distinguish SF from VCAF-1 (Xiao and Capone, 1990). Under the appropriate conditions SF significantly increases the stability of the Vmw65 dependent complex in a dose dependent manner. It is not clear, however, if this factor is essential for complex assembly because of the difficulty in completely depleting this factor from extracts containing the other essential components. The observation that ß-glucuronidase digested SF could shift mobility of VIC suggests that SF is a component of the Vmw65 dependent complex and may be composed at least partially of carbohydrates.

The significance of the SF mediated enhancement of VIC stability is still speculative. It has yet to be shown that the stability of VIC influences the ability of Vmw65 to enhance IE gene expression. In order to fully appreciate and understand the role SF plays in an HSV-1 infection a functional assay is required to measure the effect of SF on transcription levels. An *in vitro* transcription assay using a promoter containing a TAATGARAT element and the components of the Vmw65 dependent complex would permit one to directly

ascertain whether the increased stability of the VIC complex is translated into a significant increase in transcription. Unfortunately, although the AAD of Vmw65 has been shown to be quite active *in vitro* when fused to a GAL4 DNA binding domain (Chasman *et al.*, 1989), a transactivationally functional VIC complex has not yet been assembled *in vitro* (unpublished results Bilan and Capone). Thus at the present time it is not possible to measure the effect SF has on transcriptional levels.

Many distinct members of the octamer binding family of proteins have been identified in various cell types (Scholer *et al.*, 1989 a,b; Müller-Immergluck *et al.*, 1990). Vmw65 discriminates between these different proteins by virtue of differential affinities for the respective POU-homeodomains. While Oct-2 can form a complex with Vmw65, this interaction requires a 100 fold higher concentration of Vmw65 (Kristie *et al.*, 1989; Stern and Herr, 1991). A single amino acid change (ala22 to glu22) in the Oct-2 homeodomain confers, upon this protein, the ability to assemble with Vmw65 into a complex capable of activating transcription (Lai *et al.*, 1992; Pomerantz *et al.*, 1992). It is possible that factors such as SF modulate the affinity of Vmw65 (or a homologous cellular transcription regulator) for different octamer binding proteins or indeed different forms of Oct-1 by acting as adapters or co-regulators of assembly. Such a system would

thereby expand the repertoire of possible combinatorial interactions between different transcription factors. In light of this speculation, it is interesting to note that the stimulatory effect on VIC stability was most pronounced when sub-optimal concentrations of nuclear extract and Vmw65 were used in the binding reactions. Thus, the role of SF may be redundant when saturating levels of Vmw65 are available to drive complex formation more efficiently.

Kristie *et al.* (1989) have identified a HeLa factor, designated C2, that is a component of VIC. This factor does not effect the footprint pattern of VIC but does decrease the mobility of the Vmw65 containing complex in their mobility shift experiments. It is possible that the enhancement of VIC observed in the experiments described here is a result of two distinct complexes whose migration characteristics are indistinguishable in our mobility shift assay. Little is known of the characteristics of C2 and it remains to be determined if this factor is similar to SF.

In addition, there are several precedents for the regulation of transcription factor protein-DNA complex stability by low molecular weight molecules. Cavanaugh and Simons (1990 a,b) have identified a small, heat stable, protease and nuclease resistant factor that significantly stimulates DNA binding of the glucocorticoid receptor. This factor, that is similar to SF in many ways has not yet been

identified. Other low molecular weight factors have been shown to be involved in the regulation of fos-jun (Abate *et al.*, 1990a,b), CREB (Busch and Sassone-Corsi, 1990) and NFkB (Staal *et al.*, 1990) binding to DNA. The discovery of more and more of these small, regulatory molecules will add further layers of complexity and versatility to the regulation of gene expression and may signal the emergence of a separate, new class of transcription factors.

4.4   Summary of the Components Involved in the Assembly of the Vmw65 Dependent Complex

Very early in an HSV infection Vmw65 is thought to reprogram cellular gene expression by driving the assembly of complex that forms over the TAATGARAT enhancer elements. The formation of this multi-protein-DNA complex positions Vmw65 upstream of the viral IE genes facilitating the interaction of the AAD with the transcription pre-initiation complex that ultimately results in a stimulation of transcription. This complex has been shown to contain at least two cellular factors; Oct-1, a ubiquitous transcription factor that has been relatively well characterized (O'Hare and Goding, 1988) and, VCAF-1, a factor about which little is known (Xiao and Capone, 1990). The identification of SF adds a third member to the growing list of factors involved in VIC formation (Fig.

4.4.1).

The presence or absence of factors such as VCAF-1 and SF may play a role in ultimately deciding the lytic, latent or non-productive fate of an HSV infection. A defect in the assembly of VIC resulting from the absence of an essential component could predispose an infecting virus to latency in a similar manner to the *in1814* mutation (Ace *et al.*, 1989). The non-permissivity of at least one cell line (C1300 mouse neuroblastoma cells) to virus production is due to a deficiency in IE gene expression (Kemp *et al.*, 1990) that can be overcome through the over-expression of Vmw65 (Estridge *et al.*, 1990). In these cells, the TAATGARAT binding activity of Oct-2 effectively competes with Oct-1 and the assembly of the Vmw65 dependent complex. Oct-2 binding acts to inhibit IE gene transactivation (Lillycrop *et al.*, 1991; Wheatley *et al.*, 1991). It is feasible that the absence or inactivation of a VIC stabilizing factor, such as SF (or VCAF-1), may permit Oct-2 to bind up a sufficient number of the TAATGARAT elements to block the progress of a lytic infection.
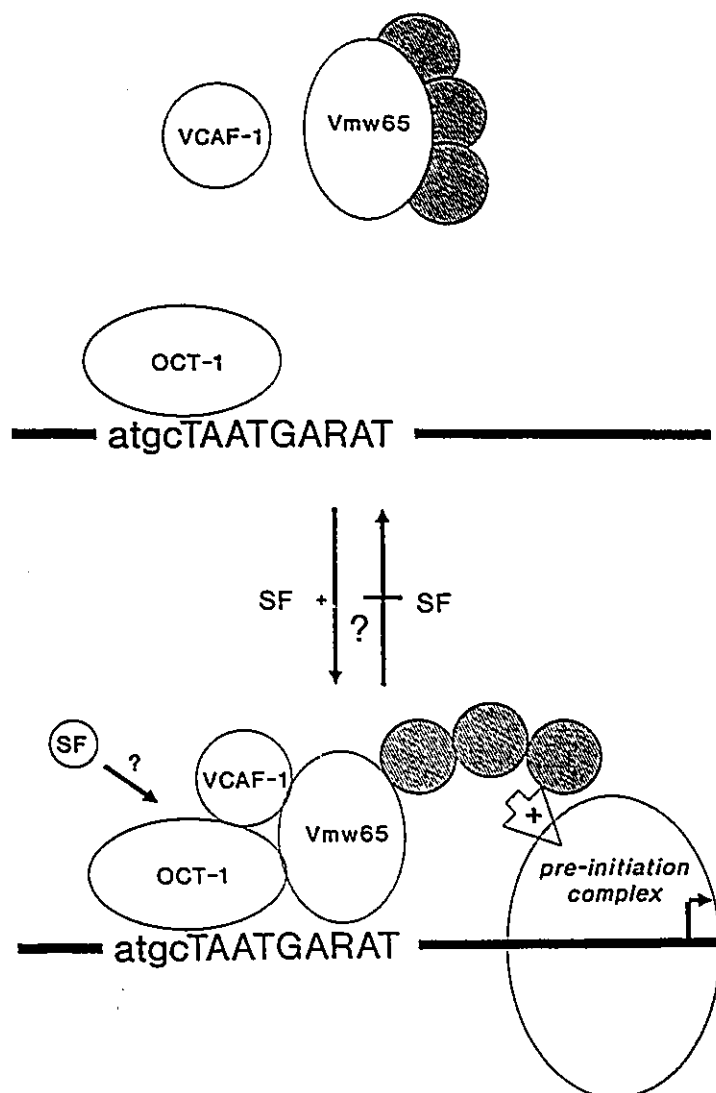
Fig. 4.4.1   A schematic diagram of the Vmw65 induced complex. Oct-1 is capable of forming a high affinity complex with the TAATGARAT enhancer element in the absence of other factors. Under similar conditions, Vmw65 interacts only weakly with the GARAT sequence and is therefore dependent upon Oct-1 and another factor, VCAF-1, for its association with DNA. A small cellular molecule, SF, significantly enhances the stability of this multi-protein-DNA complex (VIC) and may also play a role in complex assembly. The formation of VIC permits the AAD of Vmw65 (hatched domain) to interact with specific components of the transcription pre-initiation complex. This interaction facilitates the assembly of the pre-initiation complex and thereby stimulates the observed rate of transcription.

ESTABLISHMENT AND CHARACTERIZATION OF PERMANENT CELL LINES EXPRESSING VMW65

Permanent cell lines expressing Vmw65 were established to facilitate the study of this multi-functional protein. The availability of such a cell line permitted the investigation of the effect that the constitutive presence of Vmw65 had on the course of an HSV-1 infection. The course of infections with wild-type virus or a transactivation defective mutant, *in1814* were compared and the effect on infections with HSV-1 genomic DNA were examined. In a more practical sense the cell line could serve to complement the rescue of the bank of potentially lethal Vmw65 mutants into the HSV-1 genome. This could facilitate the extension of the structure-function analysis of Vmw65 and allow the study of its role as a component of the virion. Finally, the cell lines would provide yet another source of Vmw65 for work *in vitro*.
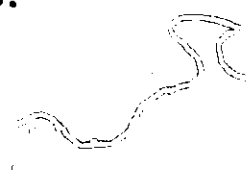
5.1   Construction of Vmw65-Expressing Cell Lines

The plasmid pZIP65 was constructed by inserting the entire coding region of Vmw65 from pMC1-B (see Fig. 3.1.2) into the Moloney murine retroviral shuttle vector, pZIPneoSV(X)1 (Cepko et al., 1984) as described in Fig 5.1.1. In this construct Vmw65 transcription would be expected to be driven at high levels by the Moloney murine leukaemia virus (MMLV) long terminal repeat (LTR). The bacterial neomycin phosphotransferase gene is located downstream of Vmw65 and is driven by the same LTR so that expression of neo is dependent upon a splicing event. This gene acts as a selectable marker, conferring resistance to the antibiotic gentamycin (G418). This plasmid also contains an SV40 origin of replication that provides a means to amplify the copy number in the presence of the large T antigen from the SV40 virus. In order to test for Vmw65 expression, Vero and BSC40 cells were transiently co-transfected with pZIP65 and the reporter plasmid p175cat, containing the chloramphenicol acetyl transferase gene driven by an ICP4 derived, IE (Vmw65 responsive) promoter. The co-transfected cells contained significantly enhanced CAT expression (40 fold) over cells transfected with p175cat and pZIPneo. This result indicated that the LTR was driving transcription and that the Vmw65 being produced was active.

Initially, it was not known how cells would react to
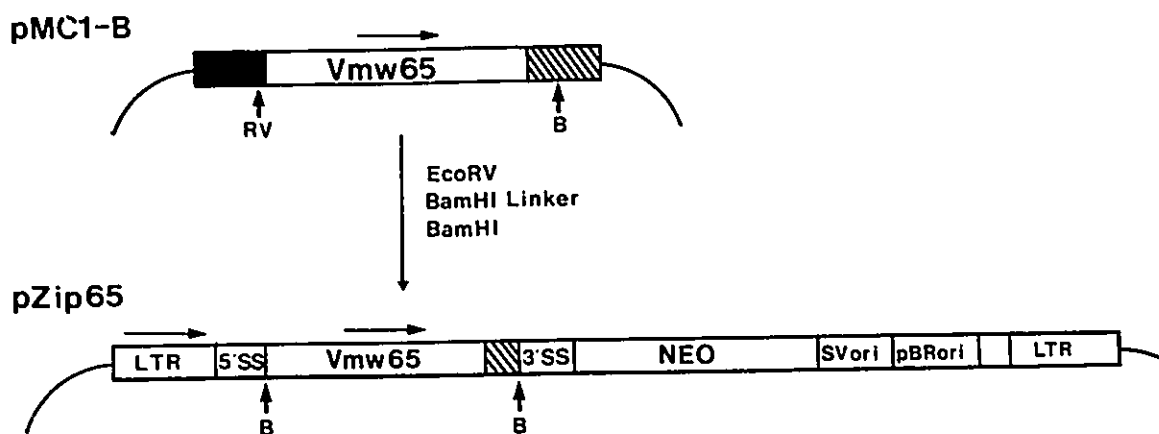
pMC1-B

pZip65

Fig. 5.1.1   Construction of pZIP65. The *EcoRV-BamHI* fragment from pMC1-B, containing the entire coding region of Vmw65, was cloned into the *BamHI* site of pZIPneo so that its expression was driven by the MMLV LTR. The adjacent 5' and 3' splice sites (SS) facilitate the expression of the downstream *neo* gene. The SV and pBR origins of DNA replication (ori) allow for plasmid amplification in tissue culture and in bacteria, respectively.

the constitutive presence of a foreign protein, especially one that activates (and squelches) transcription. In order to address this concern, attempts were made to establish a cell line that expressed Vmw65 in a temperature dependent manner. Inducible Vmw65 expression would allow the cells to be maintained and passaged in the absence of the transactivator, thereby avoiding possible detrimental effects. With this in mind, the plasmid ptsA58 was added to all transfections. This plasmid encodes a temperature sensitive version of the SV40 large T antigen (Sedivy et al., 1987). At the permissive temperature (33°C) the large T antigen would actively increase the pZIP65 copy number and subsequently Vmw65 expression. At the non-permissive temperature (39.5°C) the large T antigen would be inactive, ZIP65 would remain unamplified and Vmw65 expression would not be enhanced.

As previously stated, it was not known how various cell lines would react to the presence of Vmw65. Nor was it known if all cell lines would be equally affected. Therefore to increase the odds of finding a high expression cell line, both BSC40 and Vero cells were utilized. In addition to pZIP65 and ptsA58, another plasmid, pSVneo, was included in the co-transfection. The latter plasmid was added in some cases because it was found that pZIP65 on its own produced very few G418 resistant colonies. This was probably due to the Vmw65 insert interfering in some manner with the splicing event

required for *neo* expression. Similar effects on splicing have been reported in other studies in which this vector has been used (Korman *et al.*, 1987). Cells were transfected by the calcium phosphate method and then grown under G418 selection (800 µg/ml). After 14 days individual colonies were picked. Forty G418 resistant colonies were selected from the BSC40 plates and 20 from the Vero plates. These lines were then expanded and maintained under continuous selective pressure (400 µg/ml G418).

5.2   Screening for Vmw65 Producing Cell Lines

Individual cell lines were screened for the ability to stimulate CAT activity at 33°C from a transfected plasmid, p175cat (Fig. 5.2.1). Four lines derived from BSC40s and at least one of the Vero cell derived lines were shown to strongly enhance CAT activity compared to wild type (BSC40) cells. Initially lines III and V (from BSC40) appeared to overexpress Vmw65 in a temperature dependent manner. This effect was lost however after the first 15-20 passages. The plasmid ptsA58 contains no marker that would facilitate the selection for its presence and it may in fact have been advantageous for the cells to lose this plasmid if the over-expression of Vmw65 was even slightly detrimental to their
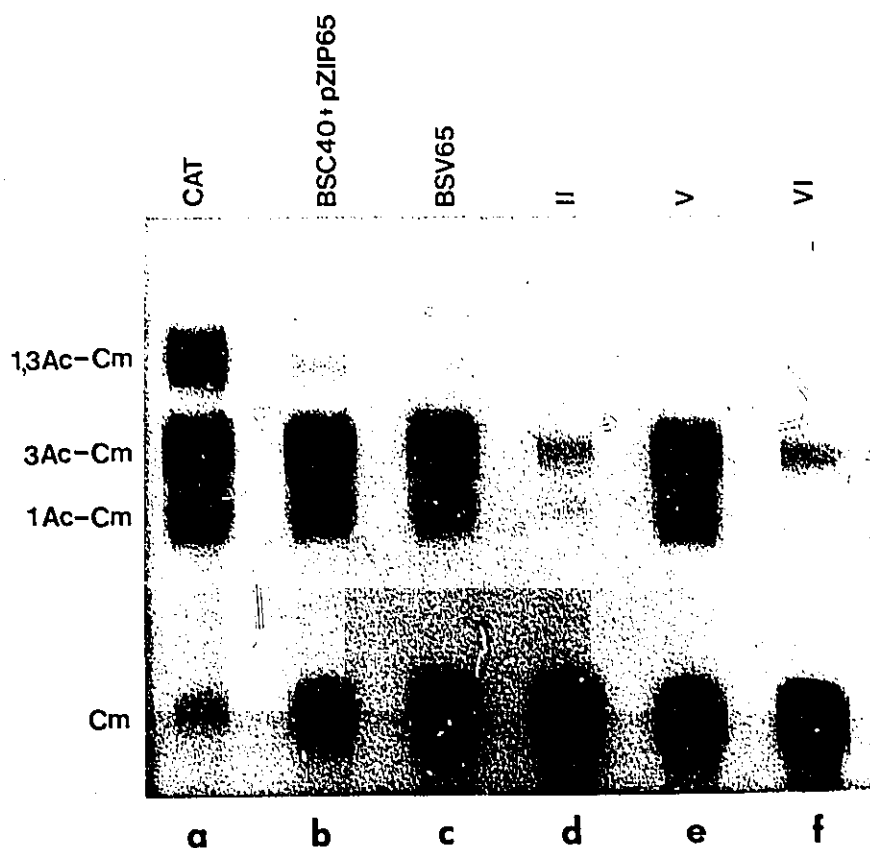
Fig. 5.2.1   Screening for 175cat activation in G418 resistant cell lines. 20 G418 resistant cell lines were transfected with 4 µg of the indicator plasmid, p175cat. Cells were maintained at 33°C and then harvested 48 hours post-transfection and CAT assays were performed. As positive controls CAT assays were carried out using the commercially obtained enzyme (lane a) and with extracts prepared from BSC40 cells that had been transfected with p175cat and pZIP65 (lane b). Of the four cell lines indicated in the figure, III and V exhibit the greatest CAT activity. Line III (renamed BSV65) was chosen for further experimentation.

continued existence. In any case, the cells when incubated at 37°C produced sufficient levels of Vmw65 constitutively to give CAT activities significantly higher (10 to 30 fold) than the parental cell line. Therefore all subsequent experiments were performed on cells grown at 37°C.

Cell line III, derived from BSC40s and renamed BSV65, was selected for further experimentation. The specificity of transcription transactivation was tested with the indicator plasmids p38Kcat, containing the cat gene driven by an HSV-2 delayed-early promoter from the gene for the small subunit of ribonucleotide reductase (an early gene which is not responsive to Vmw65 mediated trans-induction, O'Hare and Hayward (1984)) and p175cat driven by an IE promoter. The results shown in figure 5.2.2 clearly indicate that CAT activity is significantly (40 fold) higher in BSV65 cells transfected with p175cat than in the parental cell line BSC40 (lanes a and b). However, CAT activity in BSC40 and BSV65 cells transfected with p38Kcat is indistinguishable (lanes c and d). The finding that the BSV65 cell line could specifically transactivate IE promoters indicates that the cells are producing Vmw65 rather than overproducing an endogenous general enhancer of transcription. The identical activities observed upon p38Kcat transfection suggest that the two cells lines are similar with regard to their ability to take up and express plasmid DNA.
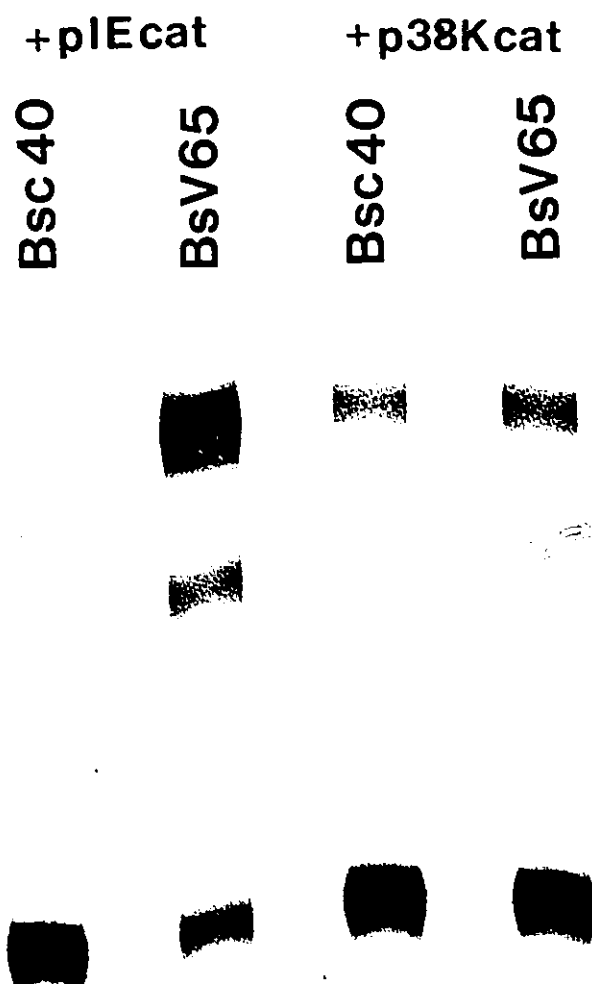
Fig. 5.2.2 Specific activation of an immediate-early promoter in BSV65 cells. BSC40 and BSV65 cells were transfected with p175cat or p38Kcat. Cells were harvested 48 hours later and CAT assays were performed. While the level of 38Kcat expression was comparable in the two cell lines, the BSV65 cells much more efficiently (40 fold) expressed 175cat.

Attempts to detect the Vmw65 protein in BSV65s by immunoflourescence, immunoprecipitating [^{35}S]-methionine labelled cell extracts or by Western blotting with the Vmw65 specific monoclonal antibody LP-1 were unsuccessful. This suggests that Vmw65 is being produced in quantities below the detection limit of these procedures or that the Vmw65 produced in this cells is unstable and is quickly degraded. Nuclear extracts produced from BSV65 were tested for the ability to form the Vmw65 dependent complex on a TAATGARAT element in a mobility shift experiment. Large amounts of the Oct-DNA complex and low, but detectable amounts of VIC were observed when 10 and 20 μg of BSV65 nuclear extract were incubated with the probe (Fig. 5.2.3, lanes e and f, respectively). These complexes have identical mobilities as the Oct-1 and VIC complexes formed with 10 μg of BSC40 extract alone (lane a) or supplemented with 2 μg of viral extract containing Vmw65 (lane b), respectively. The BSV65 VIC complex can be super-shifted with the LP-1 monoclonal antibody (lane g) though not with normal mouse serum (lane h) indicating that Vmw65 is present in the extract and is part of the observed complex. Increasing the amount of BSC40 extract present to 20 μg does not generate VIC (lane i).

a   b   c   d   e   f   g   h   i

VIC →

Oct →

VIC →

Oct →
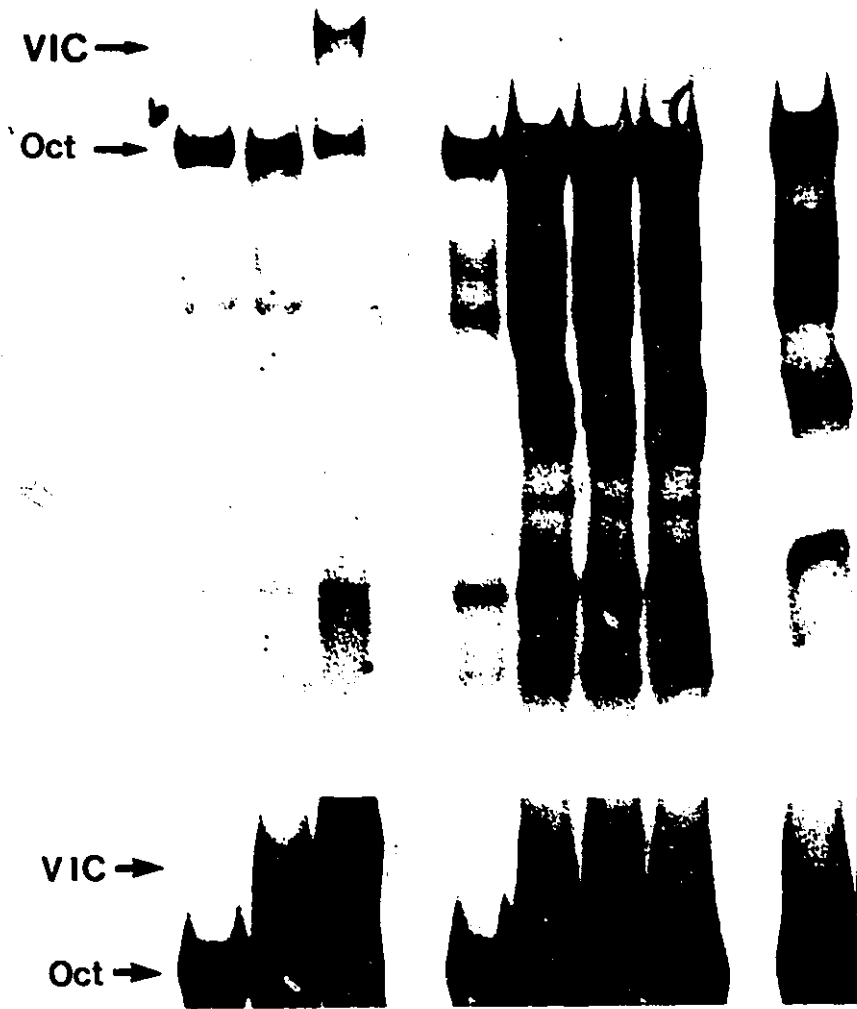
Fig. 5.2.3   BSV65 nuclear extracts can support VIC formation. An end-labelled DNA probe containing the TAATGARAT sequence was incubated with nuclear extracts from BSC40 (4 μg, lanes a-c and 8 μg, lane e) or BSV65 (4 μg, lane e and 8 μg, lanes f-h) cells. 0.1 μg of Vmw65 from purified HSV-1 virions was added to the reactions b-d. Reactions c and g also contain 1 μl of the Vmw65 specific monoclonal antibody, LP1 and lane h contains normal (pre-immune) mouse serum. Protein-DNA complexes were resolved on a non-denaturing gel. The Oct-1 (OCT) and VIC complexes are labelled accordingly. Nuclear extract from BSV65 cells is capable of forming a complex of the same mobility as VIC (compare lanes b and f). The ability of LP1 to specifically supershift this complex verifies the presence of Vmw65 (lane g). A similar complex is not detectable with increased concentrations of BSC40 extract (lane i). The figure at the bottom is a longer exposure of the same gel.

5.3   Characterization of the Vmw65-Expressing Cell Line BSV65

The above results suggest that Vmw65 is being produced in low amounts in the BSV65 cell line and is only detectable by virtue of its ability to potently stimulate transcription or by the formation of VIC as detected by the mobility shift assay. The ability of extracts from this cell line to form a specific Vmw65 containing complex on a TAATGARAT element is consistent with their ability to specifically transactivate IE promoters *in vivo*. It is possible that cell lines expressing substantially higher levels of Vmw65 were not isolated because of associated detrimental effects this may have on the cells. Vmw65 interacts with host transcription factors to stimulate cellular as well as viral gene transcription (Kemp and Latchman, 1988) and can also squelch transcription from heterologous promoters at higher concentrations (Gill and Ptashne, 1988; Triezenberg et *al.*, 1988; Berger et *al.*, 1990).

Southern blot analysis was used to determine the copy number and orientation of the Vmw65 gene in the BSV65 cell line. Genomic DNA from BSC40 and BSV65 cells was isolated and digested with *BamHI* or *ClaI*. The digested DNA was then separated by agarose gel electrophoresis along with *BamHI* digested plasmid DNA from pZIP65 corresponding to 1, 5 and 10 genomic equivalents. The DNA was transferred onto nitrocellulose and the blot was probed with a ^{32}P-labelled 1.2

Fig. 5.3.1   Southern blot analysis of BSC40 and BSV65 cells. Total DNA extracted from BSV65 (lanes e and g), BSC40 (lanes d and f) cells or 1, 5, and 10 genomic equivalents of pZIP65 were digested with BamHI (lanes a-e) or ClaI (lanes f and g). Fragments were separated on a 1 % agarose gel and blotted onto nitrocellulose. The blot was probed with a labelled 1.2 kbp SalI restriction fragment from the Vmw65 coding region of pMC1. The probe hybridized to an appropriately sized fragment of BSV65 DNA corresponding to 5 copies of the ZIP65 construct. No hybridization was observed to the BSC40 DNA (lane d). The DNA was digested with ClaI (a single cutter of pZIP65) to determine the orientation of the incorporated plasmids. The size of the band in lane g indicates that the 5 copies are positioned tandemly, in a head to tail fashion. Again no signal was observed from the BSC40 DNA.

kb *Sal*I fragment from the coding region of Vmw65. The
autoradiograph of the blot (Fig. 5.3.1) reveals that the probe
hybridized to the BSV65 DNA (lane e) but not the BSC40 DNA
(lane d). The intensity of the signal indicates that at least
5 copies of the gene are present and the mobility of the
fragment is identical to that of the *Bam*HI fragment from the
plasmid pZIP65 (lanes a-c) as expected. Digestions with *Cla*I
indicate from the fragment size that the Vmw65 gene copies in
the BSV65 cells are oriented in a head to tail fashion (lane
g) and again are not present in BSC40 cells (lane f).

5.4   Attempted Construction of a Virus Deleted of the Vmw65 ORF

One of the original objectives for the production of
a Vmw65 expressing cell line was to establish a
complementation system for the rescue of the Vmw65 mutants.
The availability of such a system would permit the examination
of the effect each mutant had on the course of a lytic
infection. The first step was to construct a virus with the
entire Vmw65 ORF deleted and replaced by a β-*gal* gene. Such a
virus would test the ability of the BSV65 cells to complement
non-functional Vmw65 mutants as well as provide a background
for marker rescue.

The plasmid pVP16β*gal* was constructed by replacing the

Vmw65 coding region of pH34 with the 3.3 kb *HincII* fragment from pMC1871 (Casadaban *et al.*, 1983) containing a ß-*gal* gene so that ß-*gal* expression is driven by the Vmw65 promoter (Fig, 5.4.1A). This plasmid is not capable of expressing Vmw65. Purified HSV-1 strain 17 genomic DNA and pVP16ßgal were co-transfected into BSV65 cells. Plaques were tested for the presence of recombinant virus, containing the ß-*gal* gene, by applying an agarose/X-Gal overlay and screening for blue plaques. Blue plaques were picked and used to infect fresh plates of BSV65 cells in an attempt to purify the recombinant virus, containing a Vmw65 null mutation. After six rounds of plaque purification contaminating, wild type virus continued to be detected. A Southern blot of the viral DNA from three different isolates indicated the presence of the desired recombinant virus intermixed with wild type virus (Fig. 5.4.1B). Therefore, the Vmw65 mutant virus was produced but could not be segregated. The inability to isolate null mutants from co-transfected BSV65 cells supports previous work indicating that Vmw65 plays an essential structural role in the virus life cycle (Halliburton and Timbury, 1976; Moss et al., 1979; Ace et al., 1988; Moss, 1989). In addition the results suggests that the BSV65 cell line does not produce sufficient Vmw65 to complement the defect of a null mutant.

More recently, a cell line has been derived from Vero cells that is similar to BSV65 in that it contains
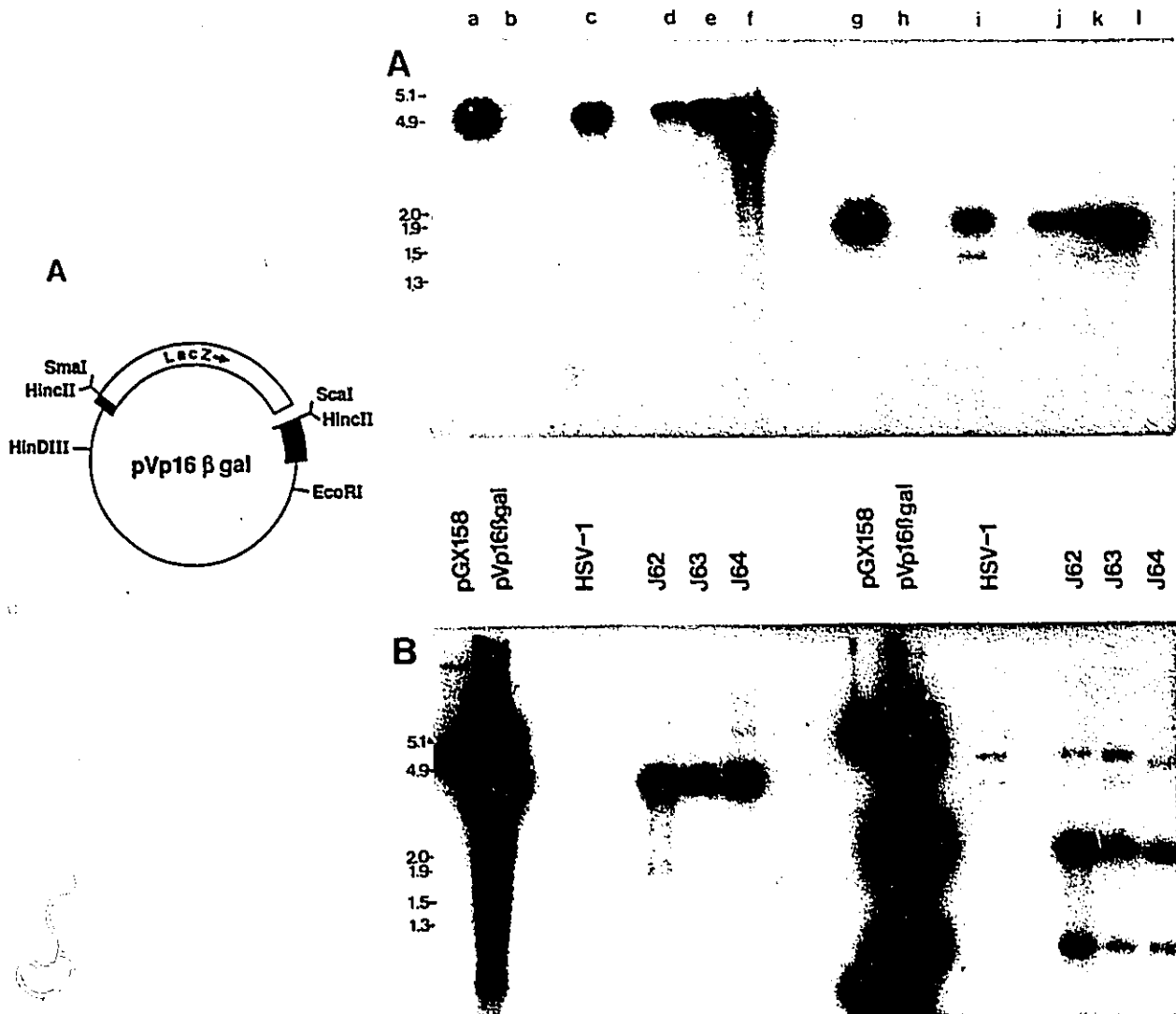
Fig. 5.4.1   Construction and attempted isolation of a virus deleted of the Vmw65 ORF. A) The 1.2 kb HincII fragment from pH34, containing a large portion of the Vmw65 ORF was replaced with the 3.3 kb SmaI-ScaI fragment from pMC1871, containing the ß-gal gene, to give pVp16ßgal. B) A Southern of HSV-1 strain 17 wild type DNA and 3 virus isolates (J62,63,64) after six rounds of plaque purification and selection for ß-gal activity. Plasmids pGX158 and pVp16ßgal are present as controls. Panel A was probed with the radiolabelled 1.2 kb SalI fragment from pMC1, containing the coding region of Vmw65. Panel B is identical to panel A but was probed with a radiolabelled BamHI fragment containing ßgal sequences. Lanes a-f contain DNA digested with BamHI and lanes g-l contain DNA digested with PstI and EcoRV. The positions of marker fragments are indicated at the side of the figure in kbp.

incorporated copies of a Moloney Murine Sarcoma Virus (MMSV) LTR driven Vmw65 gene (Weinheimer et al., 1992). Expression in these cells is inducible; they produce very little or no detectable Vmw65 in the absence of HSV infection (Smibert, personal communication); upon infection they express sufficient levels to complement a null mutation in the viral genome (Weinheimer et al., 1992). HSV-1 transactivating proteins have previously been shown to enhance transcription from the Moloney Murine Sarcoma Virus LTR (Graves et al., 1985). It can be concluded that the high level of Vmw65 needed to complement a mutant virus together with the potentially toxic effects such expression might have on a cell line require the use of an inducible Vmw65 producing cell line for marker rescue experiments.

5.5   Complementation of in1814 in BSV65 Cells

The HSV-1 mutant in1814 contains a 12 bp linker insertion mutation in the Vmw65 gene that makes it defective in the stimulation of IE gene expression (Ace et al., 1988). The in1814 mutation corresponds to H379 under the system of nomenclature used in this text. While this virus does replicate in a normal fashion when cells are infected at a

high MOI (100 or more), virus production and plating efficiency are severely reduced at low MOI's (10 or less, Ace et al., 1989). This result suggests that IE gene products must accumulate before the virus can proceed to the next stage of the infection cycle. At low MOI, and in the absence of an IE transactivator, the infection is severely impeded.

In order to determine if the constitutive presence of Vmw65 would complement the in1814 growth defect the mutant virus and wild type virus were titrated on both BSV65 and the parental BSC40 cell lines. The plating efficiency observed with in1814 was over 100 fold greater on the Vmw65 producing cells (Table 5.5.1) and nearly equivalent to the plating efficiency of wild type virus. This result suggests that the BSV65 cells are making sufficient quantities of functional Vmw65 to complement the IE transcription activation defect of in1814. The BSV65 cells, however, were incapable of complementing a virus containing a complete deletion in the Vmw65 ORF (see above). Together these results suggest that the quantity of Vmw65 required to fulfil the transactivation function is significantly less than that required to fulfil its role in virion assembly. Therefore, during the course of an in1814 infection of BSV65 cells the Vmw65 provided by the cell functions early to stimulate IE transcription. The IE gene products induce the expression of the E and L genes (that include the mutant viral Vmw65). Finally, mutant Vmw65 (and

Virus	Titer (PFU/ml) on:	
	BSC40	BSV65
Wild-type HSV-1	1.9×10^8	1.2×10^8
in1814	3.0×10^5	4.3×10^7

Table 5.5.1   Titration of wild-type HSV-1 and _in1814_ on BSC40 and BSV65 cells.

perhaps the cellular Vmw65) is packaged into the progeny virus.

5.6   Wild Type Infections are Slightly Less Efficient in BSV65 Cells

The effect of the constitutive presence of Vmw65 on the course of a wild type infection was also investigated. Plates of BSC40 and BSV65 cells were infected at an MOI of 0.1 with HSV-1 strain 17. Cells and media were collected at 12 hour intervals and virus production was determined by plaque assay on Vero cells. Surprisingly, the viral growth curves indicated that the BSC40 consistently and continuously produced 10 fold more virus than the BSV65 (Fig. 5.6.1). In addition, the infected BSV65 cells produced plaques which were smaller in size and only half the number compared to BSC40 cells (Table 5.7.1). It is possible that these effects are a direct result of the constitutive and unregulated presence of low levels of Vmw65 in the BSV65 cells which may interfere with some stage of the viral life cycle. Freidman et al. (1988) have shown that virus production is reduced 12 fold following the infection of a cell line expressing a truncated, transactivation defective form of Vmw65. The authors suggest that this is a trans-dominant effect of the defective Vmw65
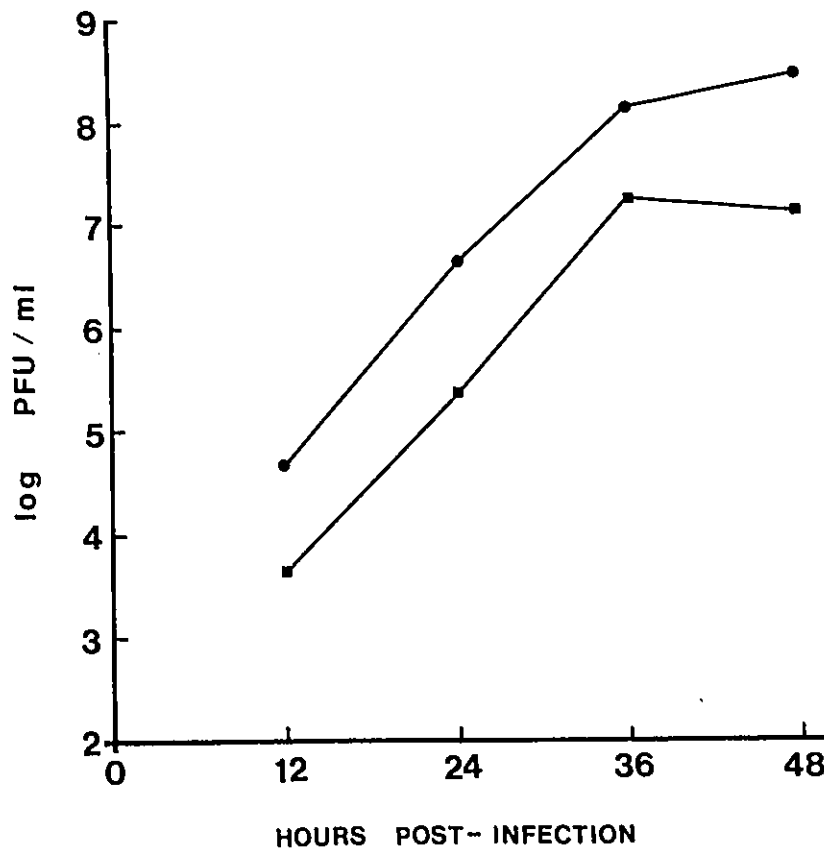
Fig. 5.6.1   Comparison of virus growth after infection of BSC40 and BSV65 cells. BSC40 (•) or BSV65 (■) cells were infected at an MOI of 0.1 with HSV-1 strain 17. After viral absorption cells were washed and incubated with 5 ml of fresh medium. Total medium and cells were harvested from individual plates at 12 hour intervals and virus titers were determined by plaque assay on Vero cells. Data shown represents the average titers from duplicate infections.

interfering with transactivation by wild type Vmw65 and therefore impeding the progress of the lytic infection. However, it was not determined that the truncated Vmw65 interfered with any stage of the infection after IE gene activation. In contrast, a Vero-derived cell line expressing Vmw65 showed no significant difference in infectivity (with HSV-1 KOS, MOI of 5) compared to the parental cells (Weinheimer et al., 1992). These cells produce Vmw65 only after infection with HSV-1 (Smibert, personal communication). Therefore, if the presence of Vmw65 interferes at a step early in infection (ie before cellular Vmw65 production), the effect may not be exhibited in this cell line. Alternatively, the differing results may be attributed to the difference in MOI (5 versus 0.1). This would suggest that at low MOI the constitutively expressed Vmw65 acts as a trans-dominant inhibitor of the HSV-1 lytic cycle. However, this inhibitory effect can be made insignificant by increasing the MOI (to 5). To more precisely determine the effect of MOI, the BSV65 cells should be infected with a range of different multiplicities.

5.7 Enhanced Infectivity of HSV-1 Viral DNA in BSV65 Cells

The fact that lytic infections result from the transfection of highly purified, protein free, HSV-1 genomic

DNA into permissive cells indicates that the virion associated proteins, including Vmw65, are not essential for the initiation of a lytic infection (Sheldrick et al., 1973; Knipe et al., 1979). The specific activity of HSV-1 genomic DNA is approximately 10^6 molecules per PFU, which is very inefficient when compared to the infectivity of intact virions (approximately 10 viral particles per PFU, Steiner et al., 1990). In addition the duration of the reproductive cycle is much longer when the infection is initiated with transfected HSV-1 DNA (Roizman and Sears, 1990). It has been suggested that this low efficiency may be due to the absence of the IE gene transactivator, Vmw65 in genomic transfection (Batterson and Roizman, 1983). This hypothesis is supported by the relative inefficiency of the Vmw65-mediated transactivation defective mutant, in1814, in infections (Ace et al., 1989). The availability of the BSV65 cell line made it possible to test what effects the presence of pre-existing Vmw65 would have on the outcome of a transfection with genomic DNA.

Purified viral DNA was transfected into both BSC40 and BSV65 cells. At 24 hour intervals post-transfection the cells and media were harvested. The viral yield of each harvest was determined by plaque assay on Vero cells. Virus was detectable on the BSV65 plates within 24 hours post-transfection after which viral yields rose steadily for the next five days (Fig. 5.7.1). Virus was not detected in the transfected BSC40 cells
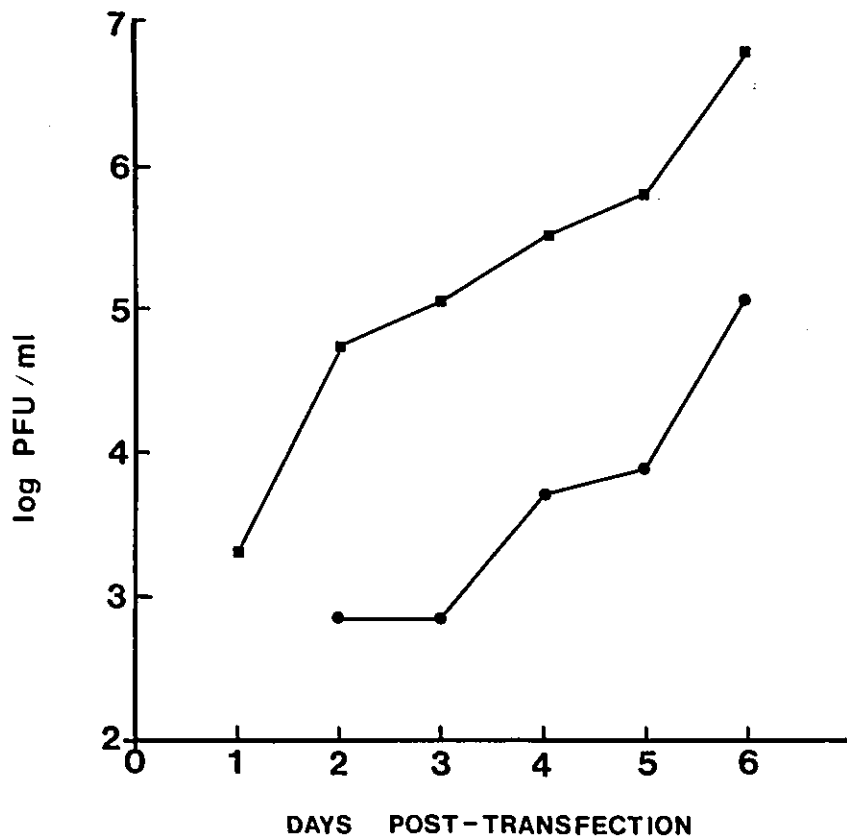
Fig. 5.7.1 Comparison of virus growth after transfection of BSC40 and BSV65 cells with HSV-1 genomic DNA. BSC40 (●) and BSV65 (■) cells were transfected with 3 μg of viral DNA. After a dimethyl sulfoxide shock cells were washed and incubated in 5 ml of medium. Total medium and cells were harvested at 24 hour intervals and virus titers were determined by plaque assay. Data shown represents the average titers of duplicate transfections.

μg of HSV-1 DNA transfected	No. of plaques ± SD[a]	
	BSC40	BSV65
0.5	1.0	4 ± 0.7
1	4 ± 1.4	10 ± 3.5
2	23 ± 2.0	28 ± 0.7
2.5	63 ± 3.5	144 ± 32
5	400 ± 28.3	584 ± 31

[a] Values are the average of two separate determinations.

Table 5.7.1   Plaque formation following transfection of viral DNA.

before the 48 hour time point. The viral yields from the BSV65 cells were 100-200 fold higher than from the parental BSC40 cells for at least the first four days. The progeny virus detected would contain Vmw65 produced late in the initial infection from the viral gene. This would sustain secondary infections on both cell lines and the virus production curves would be expected to merge.

BSC40 and BSV65 were also compared with respect to their ability to support plaque formation following genomic DNA transfection. Various amounts of viral DNA was transfected into the cells and the number of plaques formed was determined. The results show that on average 1.5-2.5 more plaques were formed per μg of genomic DNA transfected on BSV65 than on BSC40 cells (Table 5.7.1). In support of the results of the previous experiment, the plaques appeared approximately 24 hours earlier on the Vmw65 producing cell line.

Together these results suggest that IE gene activation is a limiting step in the infectivity of genomic DNA and that the presence of Vmw65 can significantly increase the efficiency of infection. Of course the role of other virion components (ie VHS, the virion host shut-off protein) cannot be excluded. Although a 1.5-2.5 increase in plaque number may not be as dramatic as the 200 fold difference observed in virus production, it may indicate that in tissue culture the presence or absence of Vmw65 has a greater effect on the

timing or pace of a productive infection rather than the ultimate outcome. This may suggest that the cells must accumulate a threshold level of IE gene products before they can proceed to the next stage of the infection. The absence of the Vmw65 transactivation function appears to predispose an incoming virus to latency (Valyi-Nagy et al., 1991).This result suggests that the fate of an infection is determined at a very early stage and the timing of IE gene activation is essential for a lytic outcome (Valyi-Nagy et al., 1991).

5.8 Enhanced Transcription of the Viral IE Genes in BSV65 Cells

If the enhanced infectivity of viral DNA in BSV65 cells is truly a consequence of their ability to stimulate IE gene transcription due to the presence of Vmw65, then one would expect to observe increased levels of the various IE transcripts in these cells. To investigate this, mRNA was isolated from BSC40 and BSV65 cells which had been transfected or mock transfected with HSV-1 genomic DNA. Because some of the IE gene products are known to autoregulate their own transcription (O'Hare and Hayward, 1985b; O'Hare et al., 1987; Everett, 1987b) cells were treated with 50 μg/ml cycloheximide beginning 30 minutes prior to transfection to block

translation. Total RNA was isolated 4 hours post transfection and blotted onto nitrocellulose using a slot blot apparatus. The blot was probed with a ^{32}P-labelled fragment from the transcribed region of the IE4 gene (Fig. 5.8.1, panel A). An identical blot was probed with ^{32}P-labelled oligo(dT) to normalize for the amount of RNA loaded in each case (Fig. 5.7.1, panel B). The bands were quantitated by densitometric scanning.

BSV65 cells contained 10 fold more IE4-specific transcript than did the BSC40 cells 4 hours post transfection and this was 100 fold over the background hybridization observed in the control mock transfected cells. As presented earlier, the transfection efficiencies of these two cells lines are indistinguishable (Fig. 5.2.2). This verifies that the BSV65 cells stimulate the production of at least one of the IE gene transcripts and correlates with the more efficient and earlier appearance of virus from this cell line. Kmetz et al. (1988) have produced a mouse cell line that expresses Vmw65 from its native promoter in addition to 2 other HSV-1 genes (UL46 and UL47). This cell line also has the ability to specifically transactivate the IE4 gene. Interestingly, infections with the in1814 mutant show greatly reduced levels of transcription of the IE genes IE0 and IE27, slightly reduced expression of IE22 but normal expression of IE4 (Ace et al., 1989). Thus the transactivation defect does not effect

	1	5	1	5
BSC 40				
BSV65				
Control				

Probe: Vmw175   poly dT
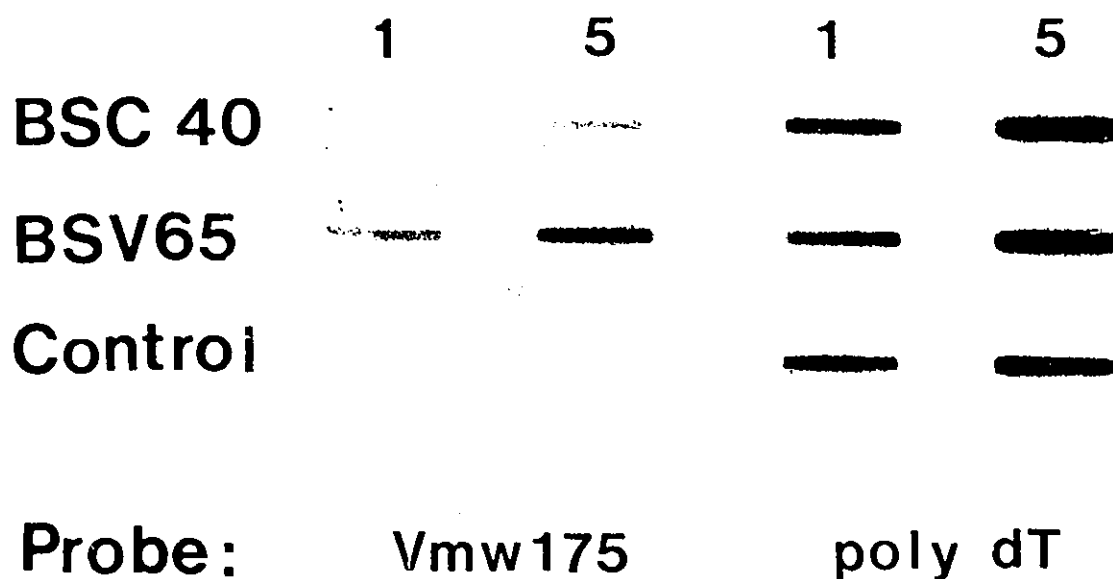
Fig. 5.8.1   Comparison of Vmw175 transcription in transfected BSC40 and BSV65 cells. BSC40 and BSV65 cells were incubated in the presence of 50 μg/ml cycloheximide 30 minutes prior to transfection (or mock transfection) with 10 μg of HSV-1 genomic DNA. Total RNA was isolated 4 hours post-transfection and 1 or 5 μg was blotted onto a nitrocellulose filter. Panel A was probed with a labelled DNA fragment from the IE4 gene (encoding Vmw175 (ICP4)). Panel B was probed with labelled oligo (dT) to normalize for the amount of RNA loaded. The control represents cells that were mock transfected.

IE4 expression in a viral infection whereas the presence of Vmw65 can enhance IE4 expression 10 fold in a genomic transfection. This apparent conflict may be rationalized if the viral DNA is presented (and treated or packaged) in different ways depending on whether it has entered the cell by infection or transfection. There is also evidence that the virion may contain copies of ICP4 (Yao and Courtney, 1989). Imported ICP4 could autoregulate its own expression and thereby negate the effects of Vmw65 (O'Hare and Hayward, 1985a). This would not occur in a transfection of naked HSV-1 genomic DNA. The examination of the other IE transcripts in transfected BSV65 cells could help explain this phenomenon. Finally, it has been recently reported that a cell cycle dependent reduction in IE4 (as well as IE0, 22 and 27) transcription is in fact detectable when synchronized HeLa cells were infected with *in1814* (Daksis and Preston, 1992). This result suggests that the requirement for Vmw65 mediated transactivation may vary depending on the phase of the cell cycle.

5.9   Summary of the Cell Line Data

A permanent cell line, designated BSV65, has been established that constitutively produces low levels of biologically active Vmw65. These cells have been shown to stimulate the transcription of at least one of the IE genes (ICP4). The IE gene activation provided by these cells is sufficient to rescue the transactivation defect in the *in1814* virus and increases the infectivity of viral DNA 200 fold.

The availability of the BSV65 cell line provides a means to study the role played by Vmw65 in viral as well as cellular gene regulation. In a more practical sense, this cell line can be utilized to increase the recovery of recombinant virus. Co-transfections of genes to be integrated and HSV-1 genomic DNA would be expected to yield more recombinant plaques, faster than the parental cell line. In addition BSV65 cells could be used in various studies as an over-expression system for IE promoter driven genes.

SUMMARY AND CONCLUSIONS

An appreciation of the methods and mechanisms by which transcription is regulated is central to our understanding of eukaryotic gene expression. The stimulation of herpes simplex virus IE gene transcription by the virion protein, Vmw65, has proven to be an excellent system for the study of transcription transactivation as well as a model for gene regulation in general. This thesis describes an investigation of the mechanisms of Vmw65 mediated transactivation and the various strategies that have been utilized to examine the different aspects of this phenomenon.

The structure-function relationship of Vmw65 was investigated through the mutational analysis of this protein. A transient transfection system was utilized to measure the transactivation function of the various mutants with respect to wild type Vmw65. The mutants, expressed in the form of protein A fusions, were tested for their ability to drive the assembly of the Vmw65 dependent, protein-DNA complex and to bind directly to DNA in the absence of other factors. Together, the results have allowed the formulation of a crude map of regions that are sensitive or insensitive to mutation

for each of the activities tested. To summarize, Vmw65 is a modular protein; the transcription activation domain (Sadowski et al., 1988) and the protein-DNA complex assembly domain (this work) can function independent of one another, and each can impart its activity to a heterologous protein. This modularity (now recognized as a common feature of transcription regulators) has facilitated the more detailed examination of each individual activity. While others have investigated the AAD, the majority of this work is concentrated upon the complex assembly function. The results indicate that Vmw65 mediated complex formation is an essential requirement for transactivation. The complex assembly function is sensitive to mutations in at least two distinct regions of the protein. One of these, the region contained between amino acids 141 and 186, is capable acting as a trans-dominant inhibitor of wild type activation in vivo and is necessary, but not sufficient, for the assembly of VIC in vitro. This information suggests that this domain interacts with a limiting component that is essential for complex formation. A future avenue of research would be to identify the ligand responsible for binding to this domain. Data from another laboratory suggests that the DNA binding function of Vmw65 may lie within this region (Stern and Herr, 1991). The further, and more specific, mutagenesis of this and other regions of interest (see Fig. 3.4.1) will more precisely map the

functional domains of Vmw65.

The direct and indirect interactions of Vmw65 with the pre-initiation complex and with other transcription factors have provided a means to identify novel factors involved in the cellular transcription regulation machinery. Examples of factors that have been discovered by virtue of their ability to interact with Vmw65 include VCAF-1 (Xiao and Capone, 1989) and at least one of the TAFs (Berger et al., 1990; Kelleher et al., 1990). An additional cellular component of the Vmw65 dependent complex was identified using cell fractionation as well as depletion and reconstitution experiments. This factor, designated SF, is a 1500-3000 Da molecule that is capable of enhancing the stability of the Vmw65 induced complex. SF activity is protease, nuclease and phospholipase resistant and may be, at least partially, composed of carbohydrates. The further characterization and the identification of this factor will be important in elucidating the role that it plays in transcription. Other small molecules have since been implicated in protein-DNA complex formation and/or transcription regulation in other systems (Cavanaugh and Simons, 1990a,b; Abate et al., 1990; Busch and Sassone-Corsi, 1990; Staal et al., 1990). Such factors may represent a family of cofactors or adaptors that function to increase the repetoire of interactions available to the components of the transcription machinery. Further investigations into the Vmw65

mediated protein-DNA complex assembly, the individual factors involved, and the role that each plays in the stimulation of transcription will be greatly assisted by the establishment of an *in vitro* transactivation assay in which this effect can be reconstituted.

A Vmw65 expressing cell line (BSV65) was isolated and characterized to facilitate the study of the physiological roles of this protein during the course of an HSV-1 lytic infection. The constitutive presence of Vmw65 enable these cells to specifically stimulate transcription from viral IE promoters. Presumably as a direct consequence, transfections of HSV-1 viral DNA into BSV65 cells resulted in a significant increase in the rate and efficiency of virus production relative to the parental cell line. BSV65 cells are capable of complementing the transactivation defect of the mutant virus *in1814* but do not express sufficient levels of Vmw65 to complement a virus deleted of the entire Vmw65 ORF. In addition to providing a means to study the multiple roles of Vmw65 this cell line continues to serve as a tool for the faster and more efficient construction of recombinant viruses.

The data presented here have added to our overall understanding of how Vmw65 stimulates transcription; specific functional domains of this protein have been identified, localized and assigned activities (at least putatively), and an additional cellular factor has been identified that plays

a role in Vmw65 dependent complex assembly. Vmw65 will continue to play a central role in the investigation and delineation of eukaryotic gene transcription and its regulation.

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