

INVESTIGATION OF PROTEIN-PROTEIN AND PROTEIN-DNA  
INTERACTIONS INVOLVED IN THE FUNCTION OF HERPES SIMPLEX  
VIRUS TRANSACTIVATOR VMW65

by

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A Thesis

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*This thesis is dedicated to my dear grandmother Huilin Zeng.*

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## ABSTRACT

The herpes simplex virus phosphoprotein Vmw65 (also referred to as VP16,  $\alpha$ TIF), strongly stimulates transcription through recognition of the *cis*-acting consensus sequence, TAATGARAT (R=purine), which is present in upstream regions of viral immediate early genes in at least one copy. However, Vmw65 has only weak intrinsic DNA binding activity. In order to execute its function as an activator, Vmw65 mediates the assembly of a multi-component complex with host cell factors that include Oct-1. The objective of this project was to utilize HSV-1 as a model system to investigate mechanisms involved in transcriptional regulation of eukaryotic genes, in particular, to elucidate how viral and cellular proteins are selected and assembled into transcriptional active complexes at the *cis*-acting sites through protein-protein and protein-DNA interactions.

Using affinity chromatography columns coupled with *E. coli* expressed protein A fused Vmw65, a novel cellular factor (referred to as VCAF-1), distinct from Oct-1, that bound directly to Vmw65 in the absence of target DNA was identified, and was found to be necessary for Vmw65-mediated complex assembly with Oct-1. This study also revealed that Vmw65 does not form a complex, directly or indirectly, with Oct-1 in the

absence of target DNA.

Some distinct properties of Vmw65 involved in generating functionally active multi-component complexes and their requirements for complex assembly and activation were investigated *in vitro* using mobility shift assays with <sup>32</sup>P-labelled oligonucleotides and purified components, as well as *in vivo* using CAT assays with mutant derivatives.

We found that Vmw65 has intrinsic DNA binding activity when used at a high concentration. This activity is sequence-specific and requires both regions 141-250 and 335-390 within the N-terminal complex forming domain (1-411). Vmw65 is able to cooperatively interact with DNA-bound Oct-1 in the absence of VCAF-1. The region containing amino acids 379-404 is required for cooperativity with Oct-1. The intrinsic DNA binding or cooperative interaction with DNA-bound Oct-1 may facilitate the assembly of multi-component complex. However, mutational analysis demonstrated that the abilities of Vmw65 to directly bind to DNA or interact with DNA-bound Oct-1 can be uncoupled from complex assembly and activation.

The independent interaction between Vmw65 and VCAF-1 may play a regulatory role in complex assembly and activation. Our study found that the sequence requirements for Vmw65 to independently interact with VCAF-1 overlaps but is distinct from that for complex formation. Furthermore, using amino acid

substitutions, we demonstrated that the direct interaction of Vmw65 with VCAF-1 is not necessarily required for the formation of the multi-component complex *in vitro*, nor for the activation of IE genes *in vivo*. Our findings suggest that more than one pathway exists in cooperative assembly of the Vmw65 induced complex, and that selective protein-protein interactions may play important roles in differentiating these pathways. This may allow HSV to adapt to various physiological conditions of infected cells and regulate its productive life cycle.

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

1. Xiao, P. and Capone, J.P. (1990) A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1. *Mol. Cel. Biol.* 10, 4974-4977.
2. Smibert, C.A., Popova, B., Xiao, P., Capone, J.P. and Smiley, J.R. (1994) Herpes simplex virus VP-16 forms a complex with the virion host shutoff protein. *J. Virol.* 68, 2339-2346.
3. Popova, B., Bilan, P., Xiao, P., Faught, M. and Capone, J.P. (1994) Transcriptional activation by DNA-binding derivatives of HSV-1 VP16 that lack the carboxyl-terminal acidic activation domain. *Virol.* 209, 19-28.
4. Shaw, P., Knez, J. and Capone, J.P. (1995) Amino acid substitutions in the herpes simplex virus transactivator VP16 uncouple direct protein-protein interactions and DNA binding from complex assembly and activation. *J. Biol. Chem.* 270, 29030-29037.

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## LIST OF ABBREVIATIONS

A	adenosine
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
C	cytosine
°C	degree celsius
CAT	chloramphenicol acetyltransferase
CFF	complex formation factor
Ci	Curie(s)
CMV	cytomegalovirus
CoA	coenzyme A
cpm	counts per minute
CTD	carboxyl terminal domain
CTK	CTD tyrosine kinase
D	dimension
Da	Dalton
dATP	deoxyadenosine triphosphate
DEAE	diethylaminoethyl

ddH <sub>2</sub> O	deionized distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E	early
EDTA	ethylenediaminetetraacetic acid
G	guanosine
g	gram
GA-	GARAT minus
GABP	GA binding protein
GTF	general transcription factor
GTP	guanosine triphosphate
HCF	host cell factor
HEPES	n-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid)
HLH	helix-loop-helix
HTH	helix-turn-helix
HSV-1	herpes simplex virus type 1
ICP	infected cell protein
IE	immediate early
IgG	immunoglobulin G
Inr	initiator
IPTG	isopropyl $\beta$ -D-thiogalactoside



k	kilo
kp	kilo base pair
L	late or litre
$\mu$	micro
m	milli
M	molar
MEM	minimal essential media
min	minute(s)
MOI	multiplicity of infection
mol	mole(s)
mRNA	messenger ribonucleic acid
MW	molecular weight
n	nano
NE	nuclear extract
NF	nuclear factor
NMR	nuclear magnetic resonance
NP40	Nonidet P-40
NTP	nucleoside 5'-triphosphate
OBP	octamer binding protein
OCTA-	octamer minus
OD	optical density
ORF	open reading frame
OTF-1	octamer binding transcription factor
p	pico

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PGE	polyethylene glycol
PFU	plaque forming unit(s)
PMSF	phenylmethylsulfonyl flouride
pol	polymerase
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SF	stimulatory factor
snRNA	small nuclear ribonucleic acid
T	thymidine
TAF	TBP associated factor
TBP	TATA binding protein
TBE	Tris-borate EDTA buffer
TBST	Tris-borate saline plus Tween 20
TE	10 mM Tris-HCl, 1 mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	transcription factor
TIF	transcription inducing factor
TK	thymidine kinase
TRF	TAATGARAT recognition factor
Tris	Tris(hydroxymethyl)aminoethane

trNA	transfer ribonucleic acid
UAS	upstream activating sequence
UL	unique long
US	unique short
UV	ultra-violet
V	volt(s)
VCAF-1	Vmw65 complex assembly factor
VHS	viral host shutoff factor
VIC	Vmw65 induced complex
VP	virion protein
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside

## INTRODUCTION

Transcriptional control of eukaryotic genes is the essential step for genetic information of an organism to be expressed in a highly regulated manner during development and in response to extracellular stimuli. In eukaryotic cells, the transcription of DNA into RNA is catalyzed by three groups of RNA polymerases: RNA polymerase I (RNA pol I), RNA polymerase II (RNA pol II), and RNA polymerase III (RNA pol III). The function of RNA pol I is to synthesize ribosomal RNAs (rRNA), which are assembled into ribosomes to carry out polypeptide synthesis. The transcripts of RNA pol III are small nuclear RNAs (snRNA) and tRNAs. rRNA, tRNA and snRNA are essential components for all protein synthesis that sustains the basic functions of a cell. The RNA pol II is responsible for the transcription of protein encoding sequences of DNA into messenger RNA (mRNA). The growth, differentiation, enzymatic reaction and signal responding of a cell, all depend upon the regulated expression of specific genes. This is mostly achieved by the transcriptional control of RNA pol II functions. Two fundamental aspects involved in this regulatory process of eukaryotic gene expression include the assembly of basal transcription machinery on the transcription start site and the selective activation of specific genes through the sequence-specific interaction of transcription factors.

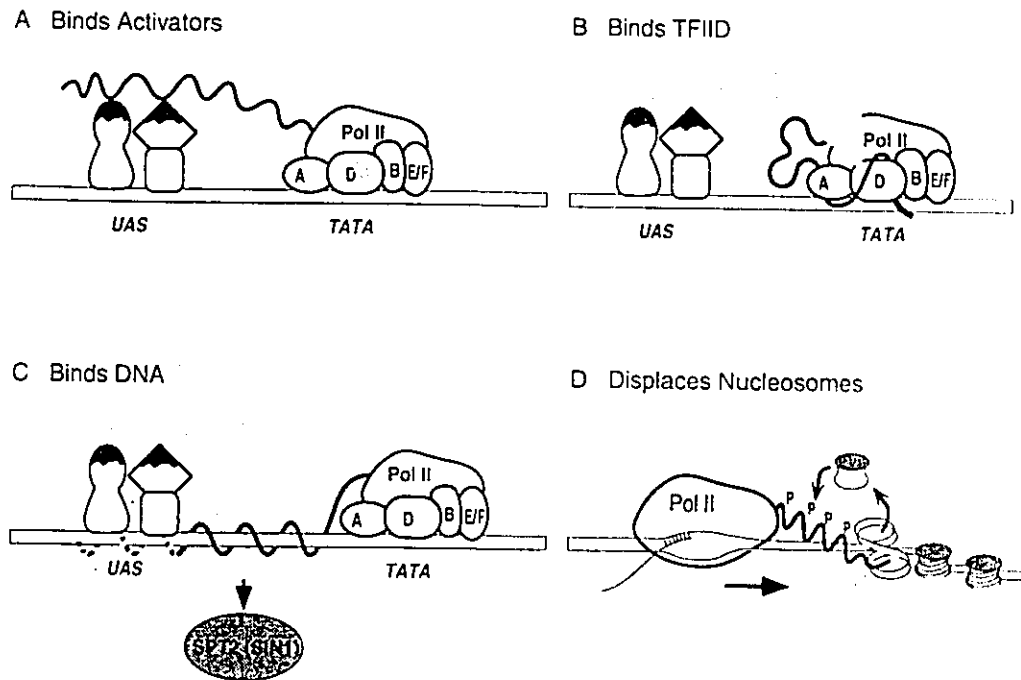
### 1.1 Initiation of transcription by RNA pol II

The promoter sequences of eukaryotic genes act as signals that direct transcription initiation by recruiting RNA pol II and transcription factors to the initiation site. The basal promoter of RNA pol II contains an initiator (Inr), which encompasses the transcription start site, and in most cases, a TATA box, which is located 25 to 30 bases upstream from the transcription start site (Matsui, *et al.*, 1981; Smale and Baltimore, 1989). To date, at least eight general transcription factors have been identified and they are referred to as TFIID, TFIIA, TFIIB, TFIIF, TFIIIE, TFIIH, TFIIG and TFIIJ *etc.* (reviewed in Conaway and Conaway, 1993). Since the first report that general transcription factors are required for RNA pol II to initiate transcription (Matsui *et al.*, 1980), these factors and RNA pol II have been extensively studied. The identification, characterization and cloning of these factors have made it possible to investigate the regulatory assembly of the multi-component initiation complex at the transcription initiation site.

The central piece of the initiation complex is RNA pol II, which is a large protein that contains 8 to 12 subunits ranging from 10 to 220 kDa (reviewed in Sawadogo and Sentenace, 1990; Greenleaf, 1992). It is still unclear if all the subunits are necessary for the enzymatic function of pol II. However, intensive studies on the largest subunit of pol II have revealed that it contains a unique carboxyl-terminal

domain (CTD), which contains the consensus sequence repeats, Tyr-Ser-Pro-Thr-Ser-Pro-Ser (reviewed in Cordon, 1990). The number of these repeats correlates to the genetic complexity of the organism, from 26-27 in yeasts, 42-44 in *Drosophila* to 52 in mammalia (Greenleaf, 1992). Deletion mutations to remove these repeats resulted in the loss of pol II functions *in vivo* and suggested that the CTD is essential for transcription initiation (Bartolomei *et al.*, 1988; Zehring *et al.*, 1988). The state of phosphorylation of the CTD is functionally significant for pol II to form the pre-initiation complex and to mediate transcriptional elongation (reviewed in Corden and Ingles, 1992). The non-phosphorylated CTD form (IIa) of pol II stably associates with pre-initiation complex perhaps by interaction with TFIID (Koleske *et al.*, 1992), while phosphorylated form (IIo) can be isolated from elongation complex (Payne *et al.*, 1989). The functions of the CTD are not yet clear though several models have been proposed (Figure 1.1.1).

In all these models, the phosphorylation of the CTD influences the preferential associations of pol II with other general transcription factors at the promoter-bound initiation complex, or with transcriptional activators. The transition from pre-initiation complex to elongation complex is also triggered by the phosphorylation of CTD (Zawel and Reinberg, 1992).



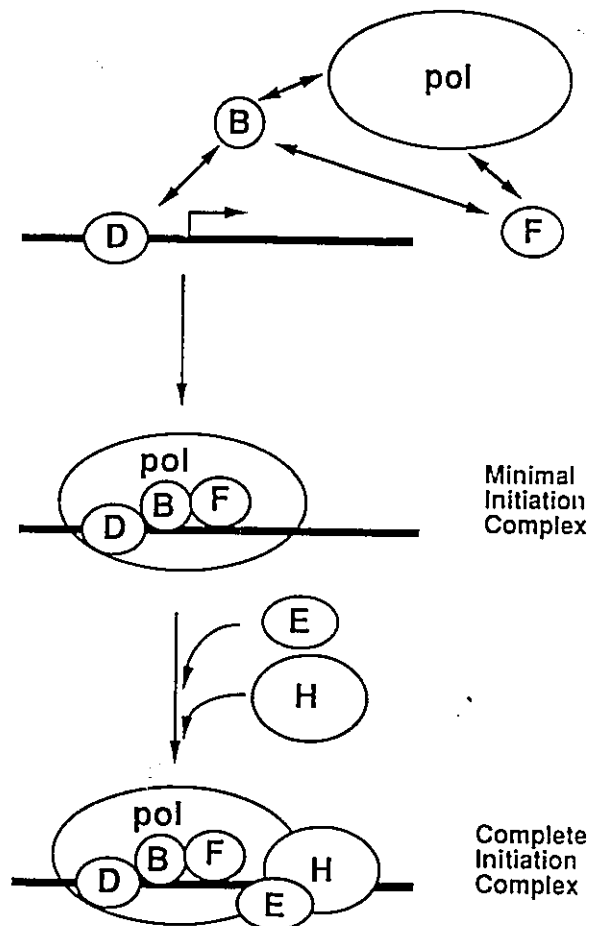
**Figure 1.1.1 Putative functions for the CTD**

(A) The CTD could directly bind to the activation domains of sequence-specific transactivators that regulate transcription from linked promoters. (B) The CTD could bind directly to one or more of the general transcription factors required for transcription initiation. The activation domains of transactivators also directly contact GTFs. (C) The CTD could bind to DNA, helping displace negative regulators such as SP2, thus facilitating interactions between activators and initiation complex. (D) The CTD functions during the elongation of RNA synthesis to displace nucleosomes in advance of elongation of RNA pol II. Adapted from Corden and Inglis, 1992.

On TATA-containing promoters, the assembly of basal initiation complex starts with the recognition of TATA box by TFIID (Sawadogo and Roeder, 1985). TFIID is a protein complex whose TATA-binding activity is attributed to a single subunit, the TATA binding protein (TBP) (Buratowski et al., 1989). TBP has been cloned from many eukaryotic cells from yeast to human (Cavallini et al., 1989; Gasch et al., 1990; Kao et al., 1990). However, in higher eukaryotic cells, the cloned TBP cannot functionally replace the endogenous form (Pugh and Tijian, 1990; reviewed in Ptashne and Gann, 1990). The recombinant TBP is able to mediate the formation of basal transcription complex but is unable to respond to activators (Peterson et al., 1990). Furthermore, the endogenous TFIID has an apparent molecular mass of over 100 kDa, while the cloned TBP has only 335 amino acid residues with a molecular mass of 38 kDa. Studies using glycerol gradient sedimentation and immunoprecipitation confirmed that mammalian TFIID is a multi-protein complex composed of at least six polypeptides (Dymlacht et al., 1991). However, the core protein, TBP so tightly associates with the TBP-associated factors (TAFs) that the dissociation of individual components can only be observed under denaturing conditions (Tanese et al., 1991; Zhou et al., 1992). Although some TAFs may function as adaptors or co-activators in activation of transcription, TFIID is usually considered a single factor for its role in transcription initiation (reviewed in Tijian and Maniatis, 1994).



The TFIID-DNA complex provides a recognition site and facilitates the association of general transcription factors with RNA pol II through protein-protein interactions (Roeder, 1991). Two other factors required for basal transcription are TFIIB and TFIIF. TFIIB directly binds to the TFIID-DNA complex probably through the interaction with the C-terminal conserved domain of TBP. This stable association of TFIIB with the TFIID-DNA complex forms the DB complex, which is required for the recruitment of RNA pol II into the initiation complex (Buratowski et al., 1989). TFIIB possesses two functional domains. The N-terminal region of TFIIB contains a zinc finger domain that is essential for recruiting RNA polymerase (Ha et al., 1993). The stable association of pol II with the DB complex is mediated by TFIIF. TFIIF contains two subunits and can form a complex with RNA pol II *in vitro*. Its small subunit is thought to bring pol II to the DB complex through the interaction with TFIIB (Flores et al., 1991; Ha et al., 1993). The complex containing TFIID, TFIIB, TFIIF and pol II has been referred to as the minimal initiation complex because it stably places on the promoter and is able to initiate transcription (Parvin and Sharp, 1993). The DBF complex is also required for the association of other general transcription factors (TFIIE and TFIIH) to form the complete initiation complex (Buratowski, 1994) (Figure 1.1.2).



**Figure 1.1.2 Model for transcription initiation complex assembly**

General transcription factor TFIID (represented by letter designations) bind to the TATA box to form TFIID-DNA complex, which facilitates the binding of TFIIB. The subsequential binding of RNA pol II is mediated by the protein-protein interactions of TFIIB and TFIIF. Pol II, and TFIID, B, F form the minimal initiation complex at the transcription start site. The formation of the complete initiation complex requires the subsequent binding of TFIIE and TFIIH. Adapted from Buratowski, 1994.

The association of TFIIA seems to increase the overall stability of the DB complex but is not essential for basal transcription (Cortes et al., 1992). Although it interacts with TBP or TFIID in the absence of DNA, TFIIA is now widely accepted as one of the TAFs rather than a general transcription factor since it mediates activator-dependent transcription (Meisterernst et al., 1991) but is not required for basal transcription (Tjian and Maniatis, 1994).

Besides the TATA element, a large number of genes transcribed by RNA pol II contain promoters without the TATA element. These promoters are called TATA-less promoters (Smale and Baltimore, 1989). Most of these genes are "housekeeping" genes because they are transcribed constitutively at a reduced rate. However, the accurate transcription of this group of genes is still modulated by all the general transcription factors including the TBP. In TATA-less promoters, the initiator is thought to direct the formation of the pre-initiation complex. RNA pol II first weakly binds to the initiator and provides a recognition sites for the general transcription factors, which subsequently associate with the Inr-bound pol II and stabilize the pre-initiation complex (Carcamo et al., 1991; Zawel and Reinberg, 1993).

## **1.2 The activation of transcription**

The accurate expression of class II genes (transcribed by RNA pol II) is modulated by transcriptional factors that

selectively interact with *cis*-acting DNA elements, in most cases, through the assembly of multi-component complexes. In lower eukaryotic genes, such as in yeast, the *cis*-acting elements are usually placed upstream from the initiation complex and called upstream activation sequences (UAS) (Guarente et al., 1982). In higher eukaryotes, the *cis*-acting elements can be located remote from the transcriptional start site and independent of orientation. Therefore, they are usually called enhancers (Banerji et al., 1981). The interaction of *trans*-acting factors may affect transcription both positively and negatively. Because the basal level of transcription in class II genes is generally low and constitutive, the activation of transcription is the major aspect of regulating eukaryotic gene expression during normal cell growth and in response to extracellular signals. Over the past decade, the mechanisms involved in activation of transcription have been intensively investigated.

Most transactivators are sequence-specific DNA binding proteins with two modular domains, a DNA-binding domain and an activation domain (Frankel and Kim, 1991). This particular structure confers both specificity and general activation functions to an activator. The DNA-binding domain of an activator plays an important role in selective activation of transcription by directing the activator to the appropriate promoter or by mediating the assembly of a transactivation complex on the promoter site. Most of the DNA-binding domains

are small, independently folded units and can be exchanged between proteins. DNA-binding domains of transactivators can be classified into different DNA-binding motifs including helix-turn-helix, zinc-binding, leucine-zipper and helix-loop-helix (reviewed in Harrison, 1991; Nelson, 1995).

### 1.2.1 DNA-binding motifs

The helix-turn-helix (HTH) DNA binding domain was first identified in prokaryotic transcription regulator  $\lambda$  repressor (Pabo and Sauer, 1984). The eukaryotic transcriptional activators that share this DNA binding motif are referred to as the homeodomain family. The structure of the HTH domain has been defined by both two-dimensional NMR (Qian et al., 1989) and X-ray crystallography (Kissinger et al., 1990). It contains a 20 amino acid segment composed of two  $\alpha$  helices, which are joined by a 120° turn. Some activators may have additional helical residues. Most of the prokaryotic HTH domains bind to DNA as a dimer, while the eukaryotic homeodomain can bind to DNA either as a monomer or as a dimer. The first helix at the amino terminus is responsible for the dimerization. The second helix at the carboxyl terminus is called the recognition helix, since it interact with the major groove of the DNA molecule and is critical for DNA binding specificity (Jordan and Pabo, 1988; Schultz et al., 1991). Besides the typical HTH motif, some extended structures have been found in this group of DNA-

binding proteins, such as a tether to connect two HTH motifs in the POU domain (Klemm *et al.*, 1994; detailed discussion in following sections); DNA-contacting extension arms (Kissinger *et al.*, 1990; Wolberger *et al.*, 1991; Feng *et al.*, 1994); and a loop to contact DNA (Harrison *et al.*, 1994).

The zinc-binding motifs include the original zinc finger and other zinc-containing DNA binding structures. They have been found in TFIIA, Zif268, Gal4 and other transcription factors. A typical zinc finger is a structure with about 30 amino acid residues, within which one  $Zn^{++}$  ion is chelated by two Cys and two His residues (Miller *et al.*, 1985; Brown *et al.*, 1985). Many proteins have two, three or more adjacent fingers. The 3-D structure of the zinc finger has been resolved by NMR (Lee *et al.*, 1989) and X-ray crystallography (Pavletich and Pabo, 1991; 1993; Fairall *et al.*, 1993). When bound to DNA, each finger lies in the major groove and contacts three base pairs.

The leucine-zipper motif is characterized by the dimerization through the basic leucine-zipper domain (bZIP). It is found in the transactivators GCN4, c/EBP, myc, jun and fos. The leucine-zipper proteins contain a region of about 30 residues rich in basic amino acids at the amino terminus followed by a 30-40 amino acid  $\alpha$ -helical coiled coil (Landschulz *et al.*, 1988). The leucine residues occur at every fourth position before other hydrophobic residues resulting in a hydrophobic surface on the helical wheel, which wraps around

each other to form a dimer. The DNA specificity is determined by the basic region (review in Alber, 1992).

Helix-loop-helix (HLH) DNA-binding motifs are found in activators that form heterodimers, such as daughterless, MyoD and myc proteins (Murre, et al., 1989). This motif is characterized by two amphipathic helices (about 15 residues) separated by a loop structure with variable size (9-20 residues). The structure of the HLH has been investigated by 2-D NMR (Schwabe et al., 1990) and X-ray crystallography (Ma et al., 1994; Ellenberger et al., 1994; Ferre-D'Amare et al., 1994). The HLH proteins without zippers, such as MyoD and E47, are called basic HLH. Their amino-terminal region forms one helix and lies in the major groove of DNA, while the carboxyl-terminal helix forms a homodimer with another molecule. Some HLH proteins have the C-terminal zipper region, which forms a two-stranded coiled-coil structure similar to that of a leucine zipper, and are called HLH zipper proteins, such as Max (Ferre-D'Amare et al., 1993) and USF (Ferre-D'Amare et al., 1994).

The structural study of DNA-binding proteins continues to expand the range and variations of the known motifs and to reveal new ones. However, the DNA-binding domains of many transcription factors, such as heat-shock proteins, SV40 large T antigen, NF- $\kappa$ b, p53, HMG-box proteins and TFIID, cannot be simply fitted into one of these motifs. Some proteins may have the DNA-binding domain of its own kind (for a recent review

see Nelson, 1995).

Other transactivators, such as Vmw65 of HSV, do not stably bind to DNA, but strongly induce the transcription of the target genes (will be discussed in detail in following sections). In order to selectively activate transcription, these factors need to be assembled into multi-component complexes at *cis*-acting DNA sites through distinct cooperative protein-protein interactions.

### 1.2.2 Structure and function of activation domains

The activation domains of eukaryotic activators stimulate high level transcription through the direct or indirect interaction with one or several GTFs or associated factors within the basal transcription machinery (reviewed in Triezenberg, 1995). These interactions are facilitated by the multiple component complexes assembled onto the promoter region, which cause the bending of the DNA molecule so that the activation domains become accessible to the components of the basal initiation complex (Roeder, 1991). Because of the lack of characteristic three-dimensional structures, the activation domains are usually grouped by the composition of their amino acids, such as the acidic activation domain (AAD) (Ma and Ptashne, 1987), the proline-rich activation domain (Mermod et al., 1989) and the glutamine-rich activation domains (Courey and Tjian, 1988).



The mechanisms involved in stimulating transcription by activation domains remain unclear. The possible functions of activation domains include repelling the repressive affects of chromatin (Paranjape et al., 1994); recruitment of the GTFs into the initiation complex (Choy and Green, 1994); enhance promoter clearance (Narayan et al., 1994); and stimulation of elongation (Yankulov et al., 1994).

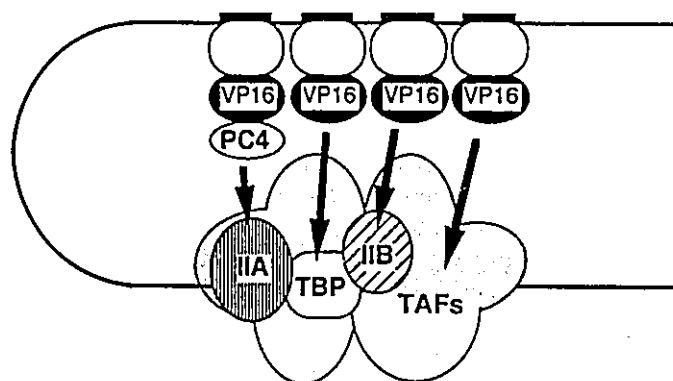
The search for the targets of activation domains has revealed that an activator, such as Vmw65, is able to bind to several GTFs, among those the interactions with TBP of TFIID (Stringer et al., 1990; Ingles et al., 1991), TFIIB (Lin and Green, 1991) and TFIIH (Xiao et al., 1994) are especially significant (Zawel and Reinberg, 1995). Furthermore, recent reports also showed the involvement of TFIIF and TFIIA in the interaction with activation domains (Zhu et al., 1994; Yokomori et al., 1994).

The interactions between activation domains and the general transcription factors TBP, TFIIB or TFIIH may be necessary for the activation of transcription. However, these direct interactions are not sufficient. First, TFIID is a stable complex containing TBP and TBP-associated factors. The efficient activation of transcription was only observed with the endogenous TFIID, but not with the purified TBP (Pugh and Tjian, 1990). It was proposed that some TAFs might be functionally linked to the activation domain during the activation (Dynlacht, 1991). Second, more recent studies have

identified some specific interactions between TAFs and different types of activation domains. The glutamine-rich activation domains of Sp1 were found to specifically interact with TAF 110 (Hoey et al., 1993; Gill et al., 1994). The AAD of Vmw65 was also found to selectively bind to TAF 40. Antibodies that block the AAD-TAF 40 interaction also prevent transcriptional activation but had no effect on basal level transcription (Goodrich et al., 1993). Furthermore, an *in vivo* study using temperature-sensitive TAF mutants has demonstrated that the association of TBP with TAFs is essential for the activation of transcription (Wang and Tjian, 1994).

### 1.2.3 Multiple targets of activators

The identification of eukaryotic coactivators and the investigation of their roles involved in multiple step activation have provided new insights of the mechanism involved in transcriptional activation. A group of cofactors have been identified recently in the laboratory of Dr. R. Roeder including PC1, PC2, PC3, and PC4 (Kretzchmar et al., 1992, 1993, 1994; Ge and Roeder, 1995). A model of multiple targets for transactivation domains and the involvement of the coactivators in the assembly of transcription activation complex has been proposed (Ge and Roeder, 1994) (Figure 1.2.1). More recently, Shykind et al. (1995) reported that a nonhistone chromosomal protein, HMG-2, acts as a coactivator in mediating activation through the interaction with the



**Figure 1.2.1 Model for multiple targets of activators**

The model depicts a partially assembled preinitiation complex of GTFs (TFIIA, TFIIB, and TFIID) thought to interact with Vmw65. Besides the direct interactions of Vmw65 activation domains with TFIIB and TFIID or a specific TAF, an additional interaction between Vmw65 and TFIIA is mediated via PC4, a coactivator. These interactions might reflect alternate or sequential interactions, or the synergism in transcriptional activation (Herschlag and Johnson, 1993). Such interactions could be involved in the recruitment or stabilization of the multiple factors, or in conformational changes that affect the function of downstream factors. Adapted from Ge and Reeder, 1994.

TFIID-TFIIA complex. Now, it is more clear that transcriptional activation is not simply achieved by direct interactions between activation domains of activators and the general factors, TFIID or TFIIB. It is rather orchestrated by the combination of these factors through a stepwise and regulated assembly of activation complexes. The interaction of activation domains to TBP or TFIID is just an important step in the process of the complex assembly.

The challenge that we have faced and are still facing is the elucidation of the mechanism by which the multiple component complexes are assembled and function. Herpes simplex virus transcriptional activator Vmw65 has provided a paradigm to study both the assembly of protein-DNA multi-component complexes on the promoter and the regulated activation of class II genes through the multi-step interaction with the basal machinery.

### 1.3 Investigation of eukaryotic class II gene expression using Herpes simplex virus type-1 as a model system

Several characteristics of herpes simplex virus type-1 (HSV-1) have made it an ideal system to study the regulated transcriptional control of eukaryotic gene expression. Over the past decade, a combination of genetic, molecular and biochemical approaches has provided us with significant insights of its gene expression pattern during the lytic life cycle of viral production. First, the natural hosts of HSV-1

are various types of human tissues (HSV-1 is also termed as human herpesvirus 1). Infection of most cell types results in a reproductive life cycle, except for neuron cells, in which latency is usually established (Roizman and Sears, 1987). HSV-1 also infects many established mammalian cell lines and provides a powerful means to investigate many features using tissue culture. Second, the patterns of gene expression associated with the lytic life cycle have been well characterized (reviewed in Roizman and Sears, 1990). Furthermore, the entire genome of HSV-1 has been cloned and sequenced (McGeoch, 1985, 1988), and most of its gene products have been identified. Finally, the transcription of HSV gene depends on the host cell RNA pol II and general transcription factors and is regulated by the combination of both viral and host cell factors.

### 1.3.1 Herpes simplex virus type-1 and gene expression

Herpes simplex virus type 1 is a member of the herpesvirus family (family: *Herpesviridae*; subfamily: *Alphaherpesvirinae*; genus: *Simplex virus*; Herpes simplex 1 virus). It has a double-stranded DNA genome packed into a icosahedral nucleocapsid. The phospholipid bilayer, with glycoproteins inserted, wraps the nucleocapsid to form an envelope and the interspace is referred to as the tegument, which contains some viral structural and regulatory proteins. The viral genomic DNA is 152 kilobase pairs (kbp) in length

and encodes about 70 genes (Morse *et al.*, 1978). The entire genome of HSV-1 has been sequenced (McGeoch *et al.*, 1985; 1988).

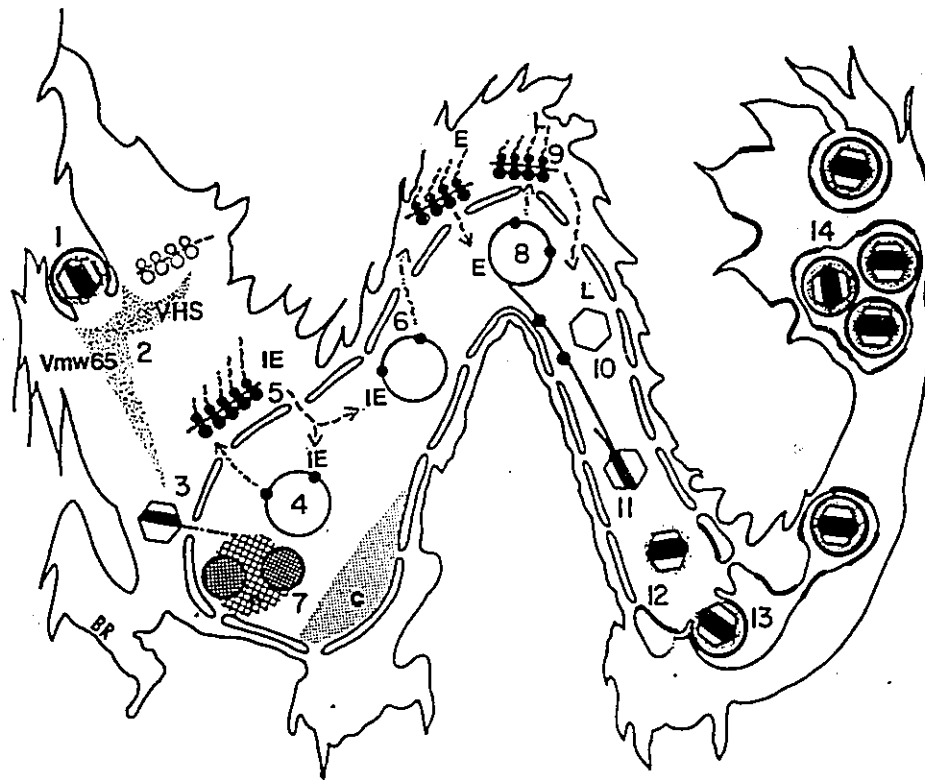
Early studies by Honess and Roizman (1974, 1975) found that the synthesis of viral proteins of HSV-1 is regulated in a temporally ordered cascade fashion. The genes of HSV can be classified into three groups,  $\alpha$ ,  $\beta$  and  $\gamma$ . The  $\alpha$  genes are expressed first upon the viral infection. The synthesis of  $\alpha$  polypeptides peak at 2-4 hours post-infection and their products are required for the expression of  $\beta$  genes. The expression of  $\beta$  genes are maximized 5-7 hours after infection. The  $\beta$  polypeptides turn off the expression of  $\alpha$  genes and induce  $\gamma$  gene expression (Watson and Clements, 1980). The  $\alpha$  genes are also referred to as immediate early (IE) genes, because their expression is prior to any viral component being synthesized. The IE gene products have been identified as infected cell polypeptides (ICPs): ICP0, ICP4, ICP22, ICP27 and ICP47, and these proteins are mostly regulatory proteins that control the later stage of HSV gene expression (reviewed in Everett, 1987 and references therein). The  $\beta$  genes are also referred to as early (E) genes, which are characterized by their enzymatic functions during viral DNA replication. The  $\gamma$  genes are also called the late (L) genes and can be further divided into  $\gamma_1$  or leaky-late and  $\gamma_2$  or true-late subgroups (Costa *et al.*, 1981). The late gene products are mostly viral structural proteins. However, some leaky-late gene products,

such as Vmw65 and VHS, play important roles in controlling the earliest events upon infection of host cells (discussed later in this thesis). The expression of the L genes reaches maximal rate 12 to 17 hours after infection (reviewed in Roizman and Sears, 1990 and references therein).

The assembly of virus particles occurs after viral DNA replication. The virion components, which are synthesized and modified in the cytoplasm of the host cell, are transported back into the nucleus where the viral DNA is packaged into nucleocapsids. The nucleocapsids then bud out from the nuclear membrane to form virus particles and result in the lysis of host cells (Figure 1.3.1).

### 1.3.2 A virion component (Vmw65) is required for the activation of HSV IE genes

Early studies by Post et al. (1981) established an approach to characterize the promoter elements of eukaryotic and viral genes. The structural sequence of a viral  $\beta$  gene, the thymidine kinase (TK), was fused to 5' non-coding sequences of  $\alpha$  genes to construct chimeric genes ( $\alpha$ -TK genes). Their study found that after infection of TK- cells, the chimeric  $\alpha$ -TK genes can be induced by superinfection with TK-virus. The induction does not require viral protein synthesis (Post et al., 1981). Batterson and Roizman (1983) further demonstrated that the efficiency of stimulating  $\alpha$ -TK gene expression by the wild type virus, UV light-irradiated virus,



**Figure 1.3.1 Schematic diagram of the HSV-1 infection cycle**  
 The progress of viral life cycle is indicated by numbers in the figure. (1) The herpes virion enters the sensory neuron and the phospholipid bilayer is fused into the plasma membrane. (2) The contents of virus are released into the cytoplasm. (3) The nucleocapsid containing genomic DNA travels to the nucleus and injects the viral DNA there. (4) Immediately upon entry into the nucleus, the viral genome circularizes and Vmw65 initiates IE gene expression. (5) The IE gene products regulate their own expression and those of downstream (E and L) genes. (6) Synthesis of E gene products that are required for viral DNA replication. (7) The cellular chromatin and nucleoli are broken down. (8) The viral DNA is replicated as a rolling circle. (9) Viral structural protein encoded by L genes are synthesized. (10) Nucleocapsids are assembled in the nucleus. (11) Viral genome is loaded into nucleocapsid. (12) The nucleocapsid matures and (13) exits the nucleus probably through the ER-Golgi membrane systems. (14) The progeny viral particles are exported to the cell surface where they are released to repeat the cycle. Adapted from Roizman and Sears, 1990.



and a temperature-sensitive mutant virus that does not release viral DNA were comparable. However, the TK-mutants of other virus families failed to induce the expression of  $\alpha$ -TK genes. These results indicated that the inducer of  $\alpha$  genes is a virion component that is present outside of the nucleocapsid.

Transient transfection experiments demonstrated that a protein encoded by HSV-1 UL48 gene was capable of activating the transcription of the ICP4 promoter controlled thymidine kinase ( $\alpha$ 4-TK) gene in cultured cells (Campbell et al., 1984; Pellett et al., 1985). UL48 is a leaky late gene and it encodes a peptide of 490 amino acids with a predicted molecular mass of 53 kDa (Dalrymple et al., 1985; Blair et al., 1987). This gene product corresponds to a previously identified HSV virion phosphoprotein VP16 (Spear and Roizman, 1972; Heine et al., 1974). Because it has an apparent molecular weight of 65 kDa determined by SDS-PAGE electrophoresis, it is also termed Vmw65 (referred to as Vmw65 in this thesis). The entire gene of Vmw65 has been sequenced (Dalrymple et al., 1985) (Figure 1.3.2).

### **1.3.3 The TAATGARAT motif is responsive to Vmw65 stimulated activation**

The cascade pattern of regulated expression of these three groups of HSV genes is due to the distinguishable structures at their promoter regions. By examining the kinetics of different  $\alpha$ TK gene products in the cell lines

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GAGCCGACCCCGCTCCGCTTCGCCCCAGACGGCCGCTGAGTGAAAACCTCCGATCCAGACATAAAGCCACACAGGGGTTCAATCCGGTGTGGGGTTCCGCTCTTCG
114
TTTCCCAATCCGACGGGGACCGGGACTGGGTGGCGGGGGTGGGTTGGACAGCCGCCCCTCGGTTCCGCTTACGTGACAGGAGCCAAATCTGGGGGAAGTCAGGGTACGGGG
128
<----->
CGCCCTCGTCCGGTTCCATAAATGGGTGGTGGCACCACGGCCCTGCTATTCCTGGGAACGGACGGGGTTCCCGCTCCCACTTCCCCCAATAAGGTCCGTCGGCTCTCTAAC
142
GCCTTTGGGGTTTTCCTTCCCGCCCGCTGGGGCTCCACACTCTCTGGCGGGGGGGGACGATCGCATCAAAAGCCGATATGCTTTTCCCGTATCAACCCCAACCCA
0
451
M D L L V D E L F A D M H A D G A S P P P P R P A G G P
28
ATG GAC CTC TTG GTC GAC GAG CTG TTT CCC GAC ATG AAC GCG GAC GGC CCT TCG CCA CCG CCC CCC CGC CCG CCC GGG GGT CCC
537
K H T P A A P P L Y A T G R L S Q A Q L M P S P F H P V
56
AAA AAC ACC CCG GCG GCC CCC CCG CTG TAC GCA ACG GGG CGC CTG AGC CAG GCC CAG CTC ATG CCC TCC CCA CCC ATG CCC GTC
621
P F A A L F H R L L D D L G F S A G P A L C T M L D T W
84
CCC CCC GGC CAC GGC CTC TTT AAC CCG CTC GTC GAC GAC TTG GGC TTT AGC GCG GGC CCC CCA TGT ACC ATG CTC GAT ACC TGG
705
K E D L F S A L P T N A D L Y R E C K F L S T L P S D V
112
AAC GAG GAT CTG TTT TCG GCG CTA CCG ACC AAC GCC GAC CTG TAC CCG GAG TGT AAA TTC CTA TCA ACG CTC GCG ACC AGC GAT GTC
789
V E W G D A Y V P E R T O I D I R A H G D V A F P T L P
140
GTC GAA TGG GGG GAC GCG TAC CTC CCC GAA CGC ACC CAA ATC GAC ATT CCG GCC CAC GGC GAC GTC GCG TTC CCT AGC CTT CCG
871
A T R D G L G L Y Y E A L S R F P H A E L R A R E E S Y
168
GCC ACC CGC GAC GGC CTC GGG CTC TAC TAC GAA ACG CAG CTC TCT CGT TTC TTC CAC CCC GAG CTA CCG GCG GAG GAG GAG GAG TAT
957
R T V L A N F C S A L Y R Y L R A S V R Q L H R Q A H N
196
CGA ACC GTC TTG GGC AAC TTC TGC TCG GCC CTG TAC CCG TAC CTG CCG GCC AGC GTC CCG CAG CTG CAC CGC CAG GCG CAC ATG
1041
R G R D R D L G E H L R A T I A D R Y Y R E T A R L A R
224
CGC GGA CGC GAT CGC GAC CTG GGA GAA ATG CTG CCG GCC ACG ATC CCG GAC AGG TAC TAC CGA GAG ACC GCT CGT CTG GCG CGT
1125
V L F L H L Y L F I L T R E I L W A A Y A E O M H R P D L
252
GTT TTG TTT TTG CAT TTG TAT CTA TTT TTG ACC CCG GAG ATC CTA TGG GCC GCG TAC GCC GAG CAG ATG ATG CCG CCG CCG CCG CTG
1209
F D C L C C D L E S W R Q L A G L F Q P F H F V H G A L
280
TTT GAC TGC CTC TGT TGC GAC CTG GAG ACC TGG CGT CAG TTG GCG GGT CTG TTC CAG CCC TTC ATG TTC CTC AAC GGA GCG CTC
1293
T V R G V P I E A R R L R E L N H I R E H L H L P L V R
308
ACC GTC CCG GGA CTA ATC GAG GCC CCG CTG CCG GAG CTA AAC CAC ATT CGC GAG CAC CTT AAC CTC CCG CTC CCG GAG CCG
1377
S A A T E E P G A F L T T P P T L H G N Q A R A S G Y F
336
ACC GCG GGT ACG GAG GAG CCA GCG GCG CCG TTG ACG ALC CCT CCC ACC CTG CAT GGC AAC CAG CCC CGC GCC TCT GGT TAC TTT
1441
H V L I R A K L D S Y S S F T T E P S E A V H R E H A Y
364
ATG GTC TTG ATT CCG GCG AAG TTG GAC TCG TAT TCC AGC TTC ACG ACC TCG CCC TCC GAG CCG GTC ATG CCG GAA CAC CCG TAC
1345
S R A P T K H N Y G S T I E C L L D L P D D D A P E E A
392
AGC CGC GCG CGT ACG AAA AAC AAT TAC GGG TCT ACC ATC GAG GGC CTG CTC GAT CTC CCG GAC GAC GAA GCC CCC GAA GAG GCG
1629
G L A A P R L S F L P A G H T R R L S T A P P T D V S L
420
GGG CTG CTG GCT CCG GCG CTG TCC TTT CTC CCC GCG GGA CAC ACG CCG AGA CTG TCG ACG CCC CCC CCG ACC GAT GTC AGC CTG
1713
G D E L H L D G E D V A H A D A L D D P D L D M L G
448
GGG GAC GAG CTC TTA GAC GGC GAG GAC GTC GCG ATG CCG CAT CCC GAC CCG CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG
1797
D G D S P C F G F T P H D S A P Y C A L D H A D F E P E
476
GAC GGG GAT TCC CCG GCG CCG GGA TTT ACC CCC CAC GAC TCC GCG CCC TAC GCG GCT CTG GAT ATG GCG GAC TTC GAG TTT GAG
1881
Q H F T D A L G I D E Y G G -
491
CAG ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGG TAG GGGGCGGACCGGACCCGATCCCGCGTCTGGGTTTTCCCGTCCCGTACCGGT
1980
-----K
TCGTATCCAAATAAACACGAGCACATACATTACAAAACCTCCGGTTCTGCTGATTAATTTGGTGGGGGAAGAAGCTAGCCAGGAGACGGGACCGCGCAACCCACT
2094
GGGCTCTGGGTTCCGGCGTGTGTATTAGCCGCTCTCCGGCCCTGCTGCTAGATTCGAAACACCGACGGCTGATGCTGTCGAGGGCGCCCGCGATAAAGGCGAGAGCG
2208
CGGACCGTTTCCGATTTGGCCGGGGCTGGGGCGCGGGTACCCCTCCCGGAGATACGCGTTTTTTTTGCGCCCGCCGCTCCCGTCCATTCGATCCGAGGGGGT
2322
CGCCGGCACCTACCCCGGCTCCATCCCGCTGTTGGGCTTTTTC TTTTGGGGTGGGAGACATCCGATAACCCCGCTATCGCCACCATGTCGCTCGCGAACCCCG
2436
GGGGCCAGGAGGCGGCAATCCACCCCGCCCGCCCTCCCGGCTGGCGGACGAGCCAGCGGGGATGGGGTGGGGTTCATGGGCTACTCGGTCGGGTCTCCCGCGGATGA
2550
CGACACGGAGCTAGAGGCTCTGGAGGAGATGGCGGGCGACGAGCCCGCCCGCTCCCGCTC
2609

```

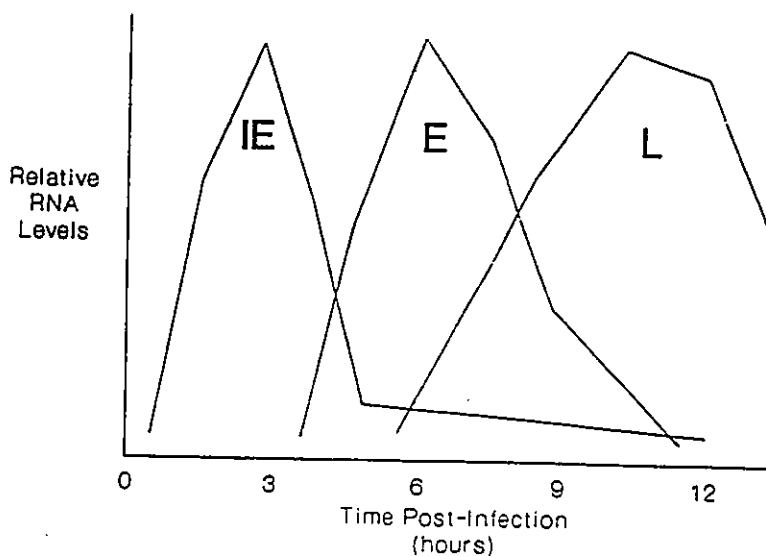
Figure 1.3.2 Nucleotide and amino acid sequence of Vmw65

The entire coding region of Vmw65 (represented by the single letter amino acid code) and the 5' and 3' untranslated region that are present in plasmid vector pMC1 are shown. Adapted from Dalrymple et al., 1985.

converted from TK- to TK+ phenotype, Post et al. (1981) investigated the relationship between  $\alpha$  promoter activities and upstream DNA elements. Several structural features of  $\alpha$  gene promoter sequences that regulate the chimeric TK genes have been characterized (Mackem and Roizman, 1982). A far upstream element, which is required for the induction of  $\alpha$ -TK genes, was found exclusively in each IE gene promoter in at least one copy (Mackem and Roizman, 1982; Cordingley et al., 1983). This sequence element is usually located 110 base pairs (bp) or farther from the transcription start site and functions independently regardless of position and orientation (Preston and Tannahill, 1984). DNA sequence analysis revealed that this consensus sequence contained a TAATGARAT (R=purine) motif (Preston et al., 1984).

The 5' sequence elements responsible for the temporal regulation of three groups have then been well characterized (Everett, 1987 and references therein) (Figure 1.3.3). All three groups of genes have the TATA box element in their promoter regions, which is essential for the basal transcription machinery. In addition to the TATA box, a GC-rich element called the GC box, which is present in many eukaryotic gene promoters and can be activated by some cellular factors like SP1, has also been found in the distal region of IE and E gene promoters. The TAATGARAT motif that is only present in IE gene promoters functions as a *cis*-acting element and is responsible for the virion component Vmw65 to

A



B

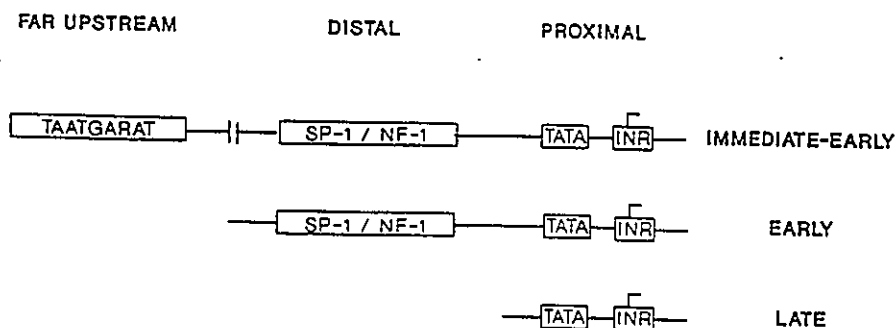


Figure 1.3.3

**A. The pattern of HSV-1 gene expression**

Three groups of HSV-1 genes are temporally controlled during the lytic cycle of infection. The relative fluctuation in the levels of viral gene transcripts are plotted against the time post-infection.

**B. The upstream elements of three groups of HSV-1 genes**

A schematic diagram showing the major sequence elements required for the regulatory expression of the IE, E and L genes.

Adapted from Everett, 1987.

activate immediate early gene expression (the assembly of the multi-component Vmw65 induced activation complex will be discussed in detail in subsequent sections). Because the lytic cycle of viral infection depends on the activation of IE genes, Vmw65 plays a central role in the regulatory control of the temporal cascade expression of HSV genes.

Besides the TAATGARAT element, another upstream *cis*-acting motif, CGGAAR, also regulates the expression of HSV immediate early genes. The direct repeats of this motif (CGGAAR)<sub>2</sub> were found in the upstream regions of ICP4 and ICP27 gene promoters (Bzik and Preston, 1986; Triezenberg et al., 1988b; apRhys et al., 1989). Each of the CGGAAR elements provides a responsive site for a cellular transcriptional activator, which was later identified from rat liver extract and designated GA binding protein (GABP) (Triezenberg et al., 1988b). This factor consists of two distinct polypeptide subunits, GABP $\alpha$  and GABP $\beta$ . Both subunits have been cloned (LaMarco et al., 1991). GABP $\alpha$  contains a region that is homologous to the DNA-binding domain of the ETS family of transcriptional factors (Karim et al., 1991). The ETS-related domain of GABP $\alpha$  recognizes the CGGAAR motif and binds the DNA with low affinity. The second subunit, GABP $\beta$ , contains four repeats (referred to as TPLH) that share sequence homology to several proteins including *drosophila* gene Notch, yeast SWI6, and Glp-1 and Lin-12 of nematodes (Breedon and Nasmyth, 1987; Michaely and Bennett, 1992). GABP $\beta$  is unable to bind to DNA

independently. However, it cooperatively interacts with GABPa and facilitates the formation of a stable DNA-binding complex on the CGGAAR element (Thompson et al., 1991).

**1.3.4 Vmw65 mediates the formation of a multi-component complex at the TAATGARAT element that is necessary for activation**

The original models suggesting that Vmw65 might activate transcription of IE genes by binding directly to the TAATGARAT *cis*-acting element in a sequence-specific manner were discounted by the fact that Vmw65 possessed no efficient DNA binding properties to either general or TAATGARAT sequences (Marsden et al., 1987). Since transfection studies also showed that Vmw65 was the only virion component required for the induction of IE gene expression, it implies that host cell factors must participate in the assembly of a multi-component DNA binding complex (McKnight et al., 1987). The first evidence of the involvement of host cell factors came from *in vitro* DNA-binding experiments that demonstrated the binding of an unidentified cellular protein to the TAATGARAT element that was required for the subsequent binding of Vmw65 (Preston et al., 1988; Kristie and Roizman, 1987; O'Hare and Goding, 1988; Trizenberg et al., 1988b). Earlier, Pruijn et al. (1986) had identified a cellular factor (nuclear factor III, NFIII) that positively regulated adenovirus DNA replication by binding to an octamer sequence homologous to

the TAATGARAT element. Because of the similarity between the octamer motif and the TAATGARAT overlapping sequences in IE gene promoters, the ubiquitous octamer-binding protein, OTF-1, was found to interact with the TAATGARAT element and form a complex with Vmw65 (Gerster and Roeder, 1988). DNA competition experiments further showed that OTF-1 was identical to the TAATGARAT recognition factor (TRF) (O'Hare and Goding 1988), which were also identified as OBP100 (Sturm *et al.*, 1987), NFIIII, and Oct-1 (Sturm *et al.*, 1988). It will be referred to as Oct-1 in this thesis.

Oct-1 is a member of the POU domain transcription factor family. The term POU is derived from transcription factors Pit-1, Oct and UNC, the three original members which share the similar DNA-binding structure later called the POU domain (Herr *et al.*, 1988). The POU domain contains two DNA contacting segments, POU-specific domain and POU-homeodomain, connected by a linker structure (Herr *et al.*, 1988; Greenstein *et al.*, 1994). Both POU segments are structurally conserved but the linker varies in length and constituents. The POU-specific and POU-homeo domains are structurally independent and both contain helix-turn-helix motifs. They contact DNA in a sequence-specific manner and the detailed interactions have been described from X-ray crystallographic data (Klemm *et al.*, 1994). The DNA target sequence for Oct-1 is ATGCAAAT. Both POU domains are placed in the major groove of the DNA molecule at opposite sides with the POU-specific domain contacting ATGC

and the POU-homeo domain binding to AAAT. The POU domain family of transcription factors are able to bind to the target DNA either as a monomer or cooperatively as a homo- or heterodimer (reviewed in Herr and Cleary, 1995).

The octamer sequence ATGCAAAT is present in various cellular gene promoters, such as histone H2B and immunoglobulin genes, as well as small nuclear RNA (snRNA) genes U1, U2, U3 and U4 promoters. It can also be recognized and bound by another highly homogenous yet unrelated octamer binding protein, Oct-2. Although Oct-1 and Oct-2 recognize the same octamer sequence indistinguishably, they display differential transcriptional regulatory properties. Oct-1 is widely expressed in almost all cell types and preferentially activates the expression of snRNA genes. However, Oct-2 is only expressed in lymphoid cells (Clerc *et al.*, 1988; Sturm *et al.*, 1988) and preferentially activates the expression of mRNA-type promoters (reviewed in Herr, 1992). Oct-1 also modulates the DNA replication of adenovirus by binding to the octamer sequence within the origin of replication (Sturm *et al.*, 1988; Coenjaerts *et al.*, 1994).

Two types of TAATGARAT sequences are present in HSV IE gene promoters. The TAATGARAT elements that overlap with the octamer sequence are referred to as type II or the (OCTA+) TAATGARAT elements (apRhys *et al.*, 1989). For instance, seven of eight ICP0 gene promoter elements contain the TAATGARAT element that overlaps with the octamer sequence to form



ATGCTAATGATAT sequence motif (apRhys et al., 1989). TAATGARAT sequences without an overlapping octamer element, are referred to as the type I or (OCTA-) TAATGARAT elements. Although Oct-1 and Oct-2 are able to bind to the (OCTA-) TAATGARAT sequence *in vitro* with less efficiency than to the (OCTA+) TAATGARAT element (Baumruker et al., 1988), neither of them alone is capable of activating the snRNA or mRNA gene controlled by an (OCTA-) promoter *in vivo*. However, in the presence of Vmw65, Oct-1 effectively activates mRNA-type (IE) genes controlled by (OCTA-) promoters, while Oct-2 is still unable to activate any IE genes (Cleary et al., 1993). These results correspond to the *in vitro* DNA-binding experiments that demonstrated that Oct-1 is much more efficient to form a complex with Vmw65, while Oct-2 lacks efficient association with Vmw65 (Kristie et al., 1989). This flexibility of the Oct-1 POU domain enables it to mediate a multi-protein-DNA complex with Vmw65 on different types of TAATGARAT upstream elements (Clear and Herr, 1995).

Although the HSV-1-specific sequence GARAT downstream from the Oct-1 binding site is not necessary for Oct-1 binding (Gerster and Roeder, 1988; Kristie and Roizman, 1988), the deletion of the GARAT element can totally abolish the induction of IE genes by Vmw65 *in vivo* and the formation of Vmw65-mediated complex *in vitro* (O'Hare et al., 1988). The results indicate that the TAATGARAT element is essential for the activation of IE gene expression through the assembly of

a multi-component *trans*-acting complex at its site (O'Hare and Goding, 1989).

The first indication that at least one other cellular factor, in addition to Oct-1, was required for the formation of the Vmw65-induced complex (VIC) was brought to light by mobility shift experiments using purified Oct-1 and Vmw65 (Gerster and Roeder, 1988). They showed that Vmw65 was capable of generating a DNA-protein complex with HeLa cell extract but not with purified Oct-1. Addition of the HeLa extract was needed for Vmw65 and purified Oct-1 to form the DNA-protein complex *in vitro*. This finding suggested that other cellular factor(s), in addition to Oct-1, was required for complex formation.

In order to use HSV-1 as a model system to investigate mechanisms of how eukaryotic genes are selectively activated by multiple factors, the first step would be to identify all components that were involved in the assembly of multi-component complexes. This thesis will describe our efforts for the identification and purification of a novel cellular factor that is required for Vmw65-induced complex assembly. This factor, designated as VCAF-1 (stands for Vmw65 complex assembly factor #1), is able to independently interact with Vmw65 and restore VIC formation *in vitro* when incubated with Oct-1 and Vmw65 (Xiao and Capone, 1990).

### 1.3.5 Vmw65 is a potent activator

Structure-function analysis reveals that Vmw65 is a modular transcriptional activator. It contains two separable functional domains. At its carboxyl terminus, Vmw65 possesses a highly acidic domain containing about 79 amino acid residues (Cousens et al., 1989). This potent domain is required for the activation function of Vmw65, because deletion mutants that have these acidic amino acid residues removed fail to stimulate transcription of IE genes (Werstuck and Capone, 1989a; Greaves and O'Hare, 1989; Cousens et al., 1989). The acidic activation domain (AAD) of Vmw65 consists of a large number of aspartic acid and glutamic acid residues. The function of the AAD is independent from that of the N-terminal domain of Vmw65, which is responsible for the formation of DNA-binding complex at the TAATGARAT element with cellular factors.

When fused to the GAL4-binding domain, the AAD of Vmw65 is able to induce the chloramphenicol acetyltransferase (CAT) reporter gene controlled by a GAL4 promoter (Sadowski et al., 1988). Although both GAL4 and Vmw65 contain the AADs, the chimeric construct of GAL4 DNA-binding domain and Vmw65 AAD gives 100 times better induction than the wild-type GAL4 (Sadowski and Ptashne, 1988). There are several other DNA-binding proteins found to have AADs, including yeast GCN4 (Hope and Struhl, 1986), yeast PUT3 (Marczak and Brandriss, 1991), and *Drosophila* and human p53 (Fields and Jang, 1990).

Among those potent activators, Vmw65 is the most potent one. Therefore, it has been used as a model to study the structure and function of activation domains.

The secondary structure of AAD is still not clear. It was first predicted to be an amphipathic alpha-helical coil with the potent amino acid residues at one side (Ginger and Ptashne, 1987). Later studies by Cress and Triezenberg (1991) using amino acid substitution to replace aspartic acid with asparagine found that these changes did not affect its function to activate  $\alpha$ -TK reporter genes *in vivo*. The study by Donaldson and Capone (1992) using biophysical approaches to determine the secondary structure also found that no specific secondary structure was present in the AAD of Vmw65. They concluded that the structure of the AAD is a random coil and its interaction with the target is in a conformationally indefinite manner.

Most of the investigations for the targets of activation domains were carried out using the AAD of Vmw65. Using affinity chromatography with AAD coupled resin, TBP of TFIID was first identified as the target site for the activation domain (Stringer et al., 1990). Further studies, (Ingles et al., 1991) demonstrated that a single amino acid substitution in TBP prevented its interaction with the AAD of Vmw65 *in vitro*. However, by the same approach, Lin and Green (1991) identified that general transcription factor TFIIB was the target for the AAD of Vmw65. They also found that the

mutants that prevented the TBP-Vmw65 interaction also disrupted the interaction between TFIIB and Vmw65. More recent studies in that laboratory demonstrated that some TFIIB mutants retained function in basal level transcription but failed to induce high level transcription in response to Vmw65 activation (Roberts *et al.*, 1993). The third GTF that binds to the AADs of Vmw65 and p53 is TFIIH (Xiao *et al.*, 1994). The interaction between TFIIH and the AAD is sensitive to the mutations that affect the activation function of the activator. Despite the association of AAD of Vmw65 with these GTFs *in vitro*, a recent study by Tansey and Herr (1995) demonstrated that the direct interaction between the AAD of Vmw65 and TBP was not relevant to the activation function of Vmw65. They showed that point mutations in a single loop of TBP that disrupted the *in vitro* interaction of TBP with Vmw65 were still capable of response to Vmw65 induced activation.

#### 1.3.6 Transcriptional synergism in activation of HSV IE genes

More recent studies by Douville *et al.* (1995) found that the efficiency of transactivation by Vmw65 for promoter with (OCTA+) TAATGARAT element was higher than that with (OCTA-) TAATGARAT element. These correspond to *in vitro* DNA binding assays, by which, they found that the Vmw65-induced complex formed on the OCTA+ probe was more stable than that formed on the OCTA- probe. However, the insertion of

neighbouring CGGAAR motifs to the (OCTA-) TAATGARAT element significantly increase the inducibility by Vmw65. Furthermore, they found that the high inducibility by Vmw65 on the (OCTA-) TAATGARAT element with nearby CGGAAR motifs was resistant to a negative regulator of IE genes, N-Oct-2, which competitively interacted with TAATGARAT elements. They suggested that this transcriptional synergism was probably due to the cooperative protein-protein interactions between Vmw65-induced complex and GABP dimeric complexes. This synergism might compensate the deficiency of Oct-1 binding to the (OCTA-) TAATGARAT element, in which half of the natural Oct-1 binding sequence, ATGC is missing, to achieve the equivalent activation for both TAATGARAT upstream elements.

#### 1.4 The project

The overall aim of this project was to investigate the transcriptional control of eukaryotic gene expression by multiple factors that form complexes at *cis*-acting DNA elements. The herpes simplex virus type 1 was chosen as a model to study the protein-protein and protein-DNA interactions involved in the regulated activation of immediate early gene expression through the stepwise assembly of multi-component complexes.

At the time I started my research project at McMaster University, Oct-1 had been identified as one of the cellular factors required for the assembly of the Vmw65-induced

complex. However, the *in vitro* protein binding experiments failed to assemble a DNA-protein complex using affinity purified Oct-1 and Vmw65 (Gerster and Roeder, 1988). This suggested that at least one cellular factor, in addition to Oct-1, was required for the assembly of the Vmw65-induced complex.

The specific goals for this project were: 1) to identify novel cellular factor(s), in addition to Oct-1, which were involved in the formation of the Vmw65-induced complex; 2) if identified, to purify this factor and characterize its biochemical properties, as well as, hopefully, to clone the cDNA for this factor; 3) to establish a system in which the stepwise assembly of Vmw65-induced complex could be studied using purified components; and 4) to investigate the requirement and functional relevance of each distinct interaction involved in the assembly of the multi-protein-DNA complex through mutational analysis of Vmw65.

We hoped the identification of a novel cellular factor would enrich our understanding to the mechanism of how HSV IE genes are selectively activated by the viron activator Vmw65 through the assembly of multi-component complexes with cellular factors. The other interesting aspects involved in Vmw65 mediated activation of viral immediate early genes also represent basics of eukaryotic gene expression (Goding and O'Hare, 1989). These include why viral gene expression is regulated by host cell factors; how protein factors are

selected and assembled onto *cis*-acting sites; how regulated assembly of protein-DNA complexes orchestrates specific gene regulatory patterns; as well as what role different *cis*-acting elements play in facilitating the assembly of distinct combinations of these factors. It was also our expectation to elucidate these basics through this project.

The thesis will be divided into five chapters. The second chapter will describe the Materials and Methods utilized in establishing assay systems for the structural and functional analysis of Vmw65. The third chapter will discuss the approaches and the results of identification of a novel cellular factor required for the assembly of the Vmw65 induced complex. The fourth chapter will discuss the establishment of an *in vitro* system to analyze the assembly of the Vmw65 induced complex using *E. coli* expressed fusion proteins, and to characterize individual interactions involved in complex assembly. The fifth chapter describes the mutagenesis study of Vmw65 to investigate the cooperative protein-protein and protein-DNA interactions and their relevancy to the complex assembly and transcriptional activation.



## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Materials

##### 2.1.1 Chemicals and reagents

<u>Name of chemical</u>	<u>Manufacturer</u>
1 Kb DNA ladder	Gibco/BRL
ampicillin (amp)	Sigma Chemical Company
acrylamide	Gibco/BRL
agarose	Gibco/BRL
bacto-agar	Difco Laboratories
bacto-tryptone	Difco Laboratories
Bio-Rad protein assay reagent	Bio-Rad
5-bromo-4-chloro-3-indoyl phosphate (BICP)	Gibco/BRL
5-bromo-4-chloro-3-indoyl $\beta$ -D-galactoside (X-gal)	Gibco/BRL
calf serum	Gibco/BRL
calf thymus DNA	Gibco/BRL
chloramphenicol	Pharmacia
CM-Sepharose	Sigma Chemical Company
Comassie Brilliant Blue	Pharmacia
CN-Br Activated Sepharose 4B	BRL
deoxynucleotide triphosphates	Pharmacia
dideoxynucleotide triphosphates	Pharmacia
DEAE dextran	Sigma Chemical Company
DEAE-Sephacel	Pharmacia
dimethyl-sulphoxide (DMSO)	BDH Chemical Company
dithiothreitol (DTT)	Boehringer Mannheim
DNA cellulose	Pharmacia
Econofluor	DuPont/NEN
ethidium bromide	Sigma Chemical Company
glutamine	Gibco/BRL
heparin-agarose	Bio-Rad
isopropylthio- $\beta$ -D-galactoside (IPTG)	Gibco/BRL
MEM non-essential amino acids	Gibco/BRL
MEM vitamin solution	Gibco/BRL
N,N'-methylene bis acrylamide	Bio-Rad
nitrocellulose	Schleicher & Schuell
Nonidet P-40 (NP-40)	United States Biochemical

Nusieve agarose	FMC Bioproducts
phenylmethylsufonyl fluoride (PMSF)	United States Biochemical
Qiagen columns and Qiaex kit	Qiagen Inc.
Salmon sperm DNA	Sigma Chemical Company
Sephadex G-50	Pharmacia
Sepharacryl 200	Pharmacia
Triton X-100	Sigma Chemical Company
Tween 20	Sigma Chemical Company

### 2.1.2 Radiochemicals

<u>Name of chemical</u>	<u>Manufacturer</u>
[3H]-acetyl coenzyme A (10 Ci/mmol)	ICN Radiochemicals
[ $\alpha$ -32P]-dATP (3000 Ci/mmol)	ICN Radiochemicals

### 2.1.3 Enzymes

<u>Name of enzyme</u>	<u>Manufacturer</u>
calf intestinal phosphatase (CIP)	Gibco/BRL, New England Biolabs
DNA polymerase I, Klenow fragment	Gibco/BRL, New England Biolabs
DNAse I	Pharmacia
lysozyme	Sigma Chemical Company
modified T7 DNA polymerase (sequenase)	United States Biochemical
restriction endonucleases	New England Biolabs, Gibco/BRL, Pharmacia, Boehringer Mannheim Pharmacia
RNase A	New England Biolabs
T4 DNA ligase	New England Biolabs
T4 polynucleotide kinase	Bio-Rad
T7 DNA polymerase (unmodified)	Gibco/BRL
Trypsin	

#### 2.1.4 Antisera

LP-1, a monoclonal antibody specifically against the N-terminal domain of Vmw65 was provided by Dr. T. Minson, Department of Pathology, University of Cambridge. Rabbit anti-Sal fragment antibody was prepared by P. Bilan of Capone Laboratory, Department of Biochemistry, McMaster University. Goat anti-mouse IgG alkaline phosphatase conjugated antibody was purchased from Bio-Rad. Donkey anti-rabbit IgG horseradish peroxidase conjugated antibody was purchased from the Amersham Life Science.

#### 2.1.5 Cell lines and viral strains

Vero and Hela S3 cell lines were obtained from the American Type-Culture Collection, Rockville, Maryland. HSV-1 strain 17 was obtained from Dr. D. Johnson, Department of Pathology, McMaster University. HSV-1 in1814 was provided by Dr. C. Preston, MRC Virology Unit, Glasgow, Scotland.

#### 2.1.6 Cloning vectors

*E. coli* expression vectors pRIT2T (Nilsson et al., 1985) and pGEX2T (Smith and Johnson, 1988) were purchased from Pharmacia. *E. coli* expression vector pMAL-c2 (Guan et al., 1987) was purchased from New England Biolabs. Cloning and *in vitro* expression vector pSPUTK was provided by Dr. D. Andrews, Department of Biochemistry, McMaster University. Eukaryotic expression vector pEVRF (Matthisa et al., 1989) was provided

from Dr. W. Schaffner, University of Zurich, Switzerland. p $\alpha$ 4CAT harbouring a chloramphenicol acetyltransferase (CAT) gene under the control of ICP4 gene promoter was constructed by Dr. J. Capone. pBSOCT-1 containing Oct-1 gene was provided by Dr. W. Herr, Cold Spring Harbour. Vectors used for site-directed mutagenesis, pTZ18 and pTZ19 (Rokeach et al., 1988) was purchased from Bio-Rad. M13/18 and M13/19 was purchased from Pharmacia.

### 2.1.7 *E. coli* strains and Phage stock

All the cloning with the exception of *lac* operator containing plasmid was performed in *E. coli* strain HB101, genotype: F $\Delta$ (mcrC-mrr) leu supE44 ara14 galK2 lacY1 proA2 rps(Str $^r$ ) xyl-5 mtl-1 recA13. Cloning of the plasmids containing the *lac* operator was performed in *E. coli* strain DH5 $\alpha$ , genotype: F'/endA1 hsdR17 (r $_k^-$ m $_k^+$ supE44thi-1 recA1 gyrA (NaI $^r$ ) relA1  $\Delta$ (lacZYA-argF) U169( $\Phi$ 80dlac $\Delta$ M15). The HB101 and DH5 $\alpha$  were purchased from Gibco/BRL. The pRIT2T constructions were transformed into *E. coli* strain N4130, genotype: F $^-$  su $^-$  his $^-$  ilv $^-$  galK $\Delta$ 8 $\Delta$ (chlD-pgl)  $\lambda$  $\Delta$ BamN+cI $_{857}$  $\Delta$ H1, purchased from Pharmacia.

M13-Vmw65ATG-1 phage stock was prepared by Dr. J. Capone.

### 2.1.8 Oligonucleotides

All the oligonucleotides mentioned in this thesis,

except for those specified, were synthesized and purified at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

#### 2.1.9 Peptides

Synthetic peptides containing portions of the amino acid sequence of Vmw65 (Vmw65 360-373 and Vmw65 374-390) were provided by Dr. Hensley, SmithKline Beecham Pharmaceuticals.

### 2.2 Analysis of Nucleic acids

#### 2.2.1 Quantitation of DNA

Fluorometer (Model TKO 100, Hoefer Scientific Instruments, San Francisco) was used for the quantitation of DNA. 2 ml of TNE buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 0.2 M NaCl) containing 1  $\mu$ g/ml Hoechst 33258 stock solution was placed in a 5 ml quartz cuvette and used as reference standard (assay buffer) to zero the fluorometer. 2  $\mu$ l of 1 mg/ml standard calf thymus DNA solution (Clontech Laboratories, Inc.) was added to the assay buffer. The fluorometer was adjusted to 200. To measure the concentration of DNA, appropriate amount of DNA sample was added to 2 ml of the assay buffer. The reading obtained from fluorometer should be between 100 and 200. If the reading was too high, further dilution of the sample was required. The concentration of DNA was then calculated by the ratio to the DNA standard. In the case of very low DNA concentration (<100 ng/ml), 0.1  $\mu$ g/ml of

Hoechst stock solution was contained in the assay buffer.

### **2.2.2 Analysis of DNA fragments by agarose gel electrophoresis**

Restriction endonuclease digested DNA and other DNA samples were separated and analyzed on agarose gels. The molecular size standard used in DNA analysis were Lambda DNA cleaved with HindIII and EcoRI, pBR322 plasmid DNA digested with MspI and 1 Kb ladder (BRL). The DNA samples were mixed with agarose gel loading buffer (0.04% bromophenol blue, 0.04 xylene cyanol, 6.7% (w/v) sucrose) and run on a agarose gel in TBE buffer ( 89 mM Tris-borate, 89 mM boric acid, 2.5 mM EDTA) containing 0.5  $\mu$ g/ml ethidium bromide. The gels were run in the same buffer at about 15 Volts/cm using a submarine gel apparatus. DNA fragments were observed and pictured under short wavelength ultraviolet light (Foto/PrepI, Fotodyne, Inc.).

### **2.2.3 DNA sequencing**

#### **1) Preparation of single stranded DNA template**

Single stranded DNA was prepared from a well isolated plaque on the *E. coli* plate infected with M13 phage containing the insertion of DNA of interest. 1 ml of overnight cultured *E. coli* cells (strain JM 101 or MV 1190) was diluted into 50 ml of fresh LB medium and dispensed 5 ml of diluted bacteria into 15 ml culture tubes (Filcon). Each tube was inoculated

with a separate plaque using a sterile loop and incubated at 37°C for 6 hours. After removing the cells by centrifugation, the culture supernatant was precipitated with 1/4 volumes of 3.5 M ammonium acetate/20% PEG 6000. The phage particles were collected by centrifugation at 17,000 g for 15 minutes and resuspended in 200  $\mu$ l of high salt buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 1 mM EDTA). The phage DNA was extracted with phenol/chloroform and precipitated with ethanol.

## 2) Denaturation of double stranded DNA for sequencing

Double stranded DNA plasmid was denatured as described by Wong et al. (1990). 5  $\mu$ g of plasmid DNA was dissolved in 16  $\mu$ l of water and 1  $\mu$ l of 4 M NaOH and 1  $\mu$ l of 4 mM EDTA was added to the solution. The denaturation was processed at room temperature for 5 minutes and 2  $\mu$ l of 2 M ammonium acetate was added to neutralize the alkaline. 60  $\mu$ l of ice cold ethanol was immediately added to the mixture and incubated in dry ice ethanol bath for 15 minutes. The DNA precipitation was pelleted by spinning for 30 minutes at 4°C and washed with ice cold 70% ethanol. The DNA was centrifuged again and dried in a rotovap for 10 minutes.

## 3) DNA sequencing

Chain termination (dideoxyl) sequencing technology was used and the reagents were provided by a Sequenase version 2 kit (United States Biochemicals). The manufacturer's suggested

procedure was followed and the annealing temperature for the sequencing primers was 65°C. The sample was run on an 8% acrylamide gel containing 8 M urea at 55 watts of constant power. The gel was dried and autoradiographed for the analysis of DNA sequences.

### **2.3 Analysis of proteins**

#### **2.3.1 Quantitation of protein**

The protein concentration was determined by Bradford method (Bradford, 1976) using the dye reagent purchased from Bio-Rad. Typically, 1 to 20  $\mu$ g of protein was dissolved in 0.8 ml water and mixed with 0.2 ml of concentrate dye reagent. After 5 minutes incubation, the absorbance at 595 nm was measured for each sample and plotted versus the concentration of a serial dilution of standard protein.

#### **2.3.2 SDS polyacrylamide gel electrophoresis**

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed as described by Sambrook et al. (1989). Mini gels were prepared in Bio-Rad gel apparatuses and big gels were prepared in Hoefer gel apparatuses. 1-2  $\mu$ g of protein sample was made up with SDS gel loading buffer (50 mM Tris, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 minutes then applied on the SDS gel. The gel was run at 150-200 watts until the dye front reached the bottom of the gel. Bio-Rad molecular weight



standard (low or high range) was run along with the protein samples. The proteins were visualized by Commassie brilliant blue or silver stain, or transferred on a nitrocellulose film for Western blotting.

### 2.3.3 Silver stain

Silver stain was performed using the Bio-Rad silver stain kit. The protocol provided by the manufacturer was followed. Immediately after SDS-PAGE electrophoresis, the gel was fixed with 40% methanol/10% acetic acid (v/v) for 30 minutes then twice with 10% ethanol/5% acetic acid (v/v) for 15 minutes. The gel was transferred to the oxidizer solution and incubated for 5 minutes then washed 3 times with deionized water until the yellow color was removed from the gel. The gel was placed in silver reagent for 15 minutes and washed with water. After washing with the developer solution for a 30 second period and removing the developer, the gel was developed in the fresh developer solution until the clear bands were observed. The developing was stopped by adding 5% acetic acid (v/v).

### 2.3.4 Immunoblot analysis

Protein samples separated by SDS-PAGE electrophoresis were transferred onto a nitrocellulose membrane (Schleicher and Schuel Inc.) using a Bio-Rad transblot apparatus. The transfer was performed at 100 mA for 12 hours in the transfer

buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol, adjusted pH to 8.3). The membrane was washed with TBST (Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 1% skim milk powder to block the active sites on the membrane. After washing twice with TBST, the membrane was incubated with primary antibody (1:1,000 dilution in TBST) for 1 hour. The membrane was washed three times with TBST for 15 minutes each. At this point, two methods were used to detect the primary antibody:

**1) Western blot AP (Alkaline phosphatase) system (Promega)**

The membrane was transferred into TBST containing the appropriate anti-IgG-AP conjugate (1:7,500 dilution) and incubated for 30 minutes. The nitrocellulose was washed three times with TBST for 10 minutes each to remove the unbound antibody. The membrane was damp dried on Whatman papers and transferred to the color development solution (to every 10 ml of AP buffer (100 mM Tris\_HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>), 66  $\mu$ l NBT substrate was added, mixed well, then 33  $\mu$ l of BCIP substrate was added and mixed again). The reactive areas would turn purple with in 1-15 minutes and the color development was allowed to continue until the desire color intensity was achieved. The reaction was stopped by washing the membrane with water.

## **2) ECL (Enhanced Chemiluminescence) Western blot (Amersham)**

The membrane was incubated with TBST containing appropriate anti-IgG HRP (horseradish peroxidase) antibody (1:5,000 dilution) for 30 minutes. The membrane was washed 3 times with TBST for 15 minutes each then once with TBS (Tris-HCl, pH 7.6, 137 mM NaCl). After draining the excess buffer, the membrane was placed in a fresh container. The detection solution (1:1 mix of detection solution 1 and 2) was added (sufficient volume to cover the membrane) and incubated precisely for 1 minute. The membrane was immediately drained off the excess detection solution and wrapped into a Saranwrap. A DuPont film was put onto the membrane in a dark room to expose for 15 seconds. The film was developed immediately to adjust the time for second exposure.

## **2.4 Analysis of protein-DNA interactions**

### **2.4.1 Preparation of gels for mobility shift assays**

Gels for mobility shift assays were prepared using Hoefer gel apparatuses. 3.5-4% non-denatural polyacrylamide gels (30:0.8 ratio of acrylamide to bis-acrylamide) in low ionic strength (in 0.25 X TBE) were used for the mobility shift assays. All gels were pre-run in 0.25 X TBE for at least one hour.

#### 2.4.2 Preparation of radio labelled probe for mobility shift assays

Synthetic oligonucleotides for mobility shift assays were annealed by heating to 95°C and then slowly cooling to room temperature over a 5 hour period in a buffer containing 50 mM Tris-HCl, pH 8.0 and 10 mM MgCl<sub>2</sub>. The labelling reaction contained 15 pmole of annealed oligonucleotide, 100 μCi of α-<sup>32</sup>P-dATP, 20 nM of each dGTP, dCTP and dTTP, and 5 units of Klenow. The reaction was carried out at room temperature for 2 hours and chased with 20 nM of dATP for 10 minutes. Two methods were used to purify the radio labelled probe.

1) The probe was separated on a 4% Nuseive agarose gel and continually run onto a S&S N45 DEAE ion exchange nitrocellulose paper inserted in front of the desired band. The probe was eluted from the S&S N45 paper by incubating in 1.25 M NaCl at 68°C for 30 minutes. The eluent was spun for 15 minutes. The probe was precipitated by adding ethanol to the supernatant and resuspended in 100 μl of water.

2) Bio-spin chromatography columns (Bio-Rad) were used to purify the probe. The Bio-spin column pre-equilibrated with 0.15 mM NaCl, 17.5 mM sodium citrate were placed in a collection tube and centrifuged for 2 minutes at 1,100 g to remove the buffer. The labelled probe (added volume to 100 μl) was applied onto the column dropwise. The column was then placed in a clean tube and centrifuged 4 minutes at 1,100 g to collect the probe.

### 2.4.3 Mobility shift assays

Protein-DNA mobility shift assays were performed using the procedure developed by Fried and Crothers (1981), Garner and Revzin (1981) and modified by Strauss and Varshvsky (1984). The standard reactions were carried out in 15  $\mu$ l containing 5 mM Hepes, pH 7.9, 0.1 mM DTT, 0.5 mM EDTA, 25 mM KCl, 4  $\mu$ g of competitor DNA (a 1:2 mixture of salmon sperm DNA and poly(dI-dC)). Hela nuclear extract, fractions of chromatography and purified proteins were added to the reaction as indicated. protein concentration in each reaction was normalized with BSA. Reaction mixtures were preincubated for 5 minutes at room temperature before the addition of 0.15 pmole of  $^{32}$ P labelled probe. Unless specified otherwise, the probe used in mobility shift assays was 5'-GTACCCGTGCATGCTAATGATATTCTTT, which contained the promoter-proximal TAATGARAT element from the HSV-1 ICP0 gene. Binding reactions were incubated at 30°C for 20 minutes. To those, 1  $\mu$ l of loading buffer (20% glycerol, 0.1% Bromophenol blue, 0.1% xylene cyanol) was added. The protein-DNA complexes were resolved on a 3.5% polyacrylamide gel described above. The gels were run at 220 volts until the dye front reached 1 cm from the bottom, then dried on a vacuum gel dryer (Bio-Rad).

## 2.5 Manipulation of DNA

### 2.5.1 Preparation of competent cells

The protocol to prepare competent cells capable of

taking up plasmid DNA relies on the disruption of cell wall caused by high concentration of calcium ions. A well isolated colony from the appropriate bacteria strains (i.e. HB101, DH5 $\alpha$ , MV1109) is inoculated into 20 ml of LB medium and grown overnight at 37°C with shaking. 100 ml of LB medium was inoculated with the overnight culture to give an initial OD<sub>600</sub> of 0.1 and continue to grow at 37°C until the culture reaches an OD of 0.5. The cells were harvested by centrifugation at 4°C and resuspended in 20 ml of RF-1 (100 mM RbCl, 50 mM MnCl 30 mM KOAc, 10 mM CaCl<sub>2</sub> and 15% glycerol adjusted to pH 5.8 with 0.2 M HAc). The cells were incubated on ice for 1 hour then gently pelleted. They were gently resuspended in 10 ml pre-chilled RF-2 (10 mM MOPS, 10 mM RbCl, 75 mM CaCl<sub>2</sub> and 15% glycerol adjusted to pH 6.8 with NaOH) until a smooth suspension was obtained. After aliquoting into 200  $\mu$ l, the cells were freshly frozen in liquid nitrogen and stored at -80°C.

The preparation of competent cells from *E. coli* strain CJ-236 (*dut*<sup>-</sup>, *ung*<sup>-</sup>) was slightly different from above and will be discussed in the section of oligonucleotide directed mutagenesis.

#### 2.5.2 Transformation of *E. coli*

According to the type of bacteria strain, 50 to 200  $\mu$ l of competent cells was placed in a pre-cold Eppendorf tube. If frozen competent cells were used, thaw them on ice.

Approximately 10-100 ng of DNA from a ligation reaction or plasmid was added to the competent cells. The cells were incubated on ice for a period of 45 to 60 minutes then heat shocked at 42°C for 2 minutes. Return the cells to ice for 5 minutes and add 1 ml of 2YT medium. The cells were grown for 1 hour at 37°C to allow for the expression of the ampicillin resistant gene. Varying amounts of the cells (25 to 200  $\mu$ l) were spread on plates containing 2YT, 1.5% bacto-agar, 50  $\mu$ g/ml ampicillin, (and 50  $\mu$ l of 2% X-gal and 10  $\mu$ l of 100 mM IPTG if the plasmid contains a *lac* operator). The plates were incubated overnight at 37°C.

### 2.5.3 Preparation of plasmid DNA

Small scale of plasmid DNA for the purpose of screening was prepared with the mini-preparation method called "10 minutes mini-prep." adapted from Dr. D. Andrews, Department of Biochemistry, McMaster University. Briefly, 1.4 ml of overnight bacteria culture was placed in an Eppendorf tube and pelleted with a microfuge for 30 seconds. Remove the supernatant but leave 50  $\mu$ l of medium and resuspend the cells in the medium. 300  $\mu$ l of TENS solution (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0, 0.1 N NaOH, 0.5% SDS) was added to the suspension. Vortex 2 to 5 seconds until the mixture becomes sticky. Add 150  $\mu$ l of 3M sodium acetate, pH 5.2 and vortex 2 to 5 seconds to mix. The mixture was spun for 2 minutes and the supernatant was transferred to a fresh tube. 900  $\mu$ l of absolute ethanol was added and mixed well by inverting.

DNA/RNA was pelleted with 2 minutes of spinning. After discarding the supernatant and rinsing the pellet with 1 ml 70% ethanol, the pellet was air dried and resuspended into 25 to 50  $\mu$ l of TE buffer containing 1  $\mu$ g/ml of DNase free-RNase A.

Larger scale of plasmid for cloning was prepared on Qiagen columns (P-20, P-100, and P-500) using the protocols provided by the manufacture. Before applying on the Qiagen column, various amount of bacterial culture was grown overnight in 2YT medium containing 50  $\mu$ g/ml ampicillin at 37°C. The cells were harvested by centrifugation, resuspended into buffer P1 (100  $\mu$ g/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA, pH 8.0) and lysed with buffer P2 (200 mM NaOH, 1% SDS). Buffer P3 (3.0 M KOAc, pH 5.5) was added to precipitate the denatured proteins, chromosomal DNA and cellular debris. The precipitation was removed by high speed (>30,000xg) centrifugation. The supernatant was applied on the Qiagen column pre-equilibrated with buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0 15% ethanol, 0.15% Triton X-100). The contaminants were removed by washing the column twice with buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol). The plasmid DNA was eluted with buffer QF (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5 15% ethanol) and precipitated with 0.7 volumes of isopropanol. After the DNA was pelleted by centrifugation, it is washed with 70% ethanol, air dried and resuspended into an appropriate amount of TE buffer (pH 8.0). The concentration of the plasmid DNA was determined by a



fluorometer (Model TKO 100, Hoefer Scientific Instruments, San Francisco).

#### 2.5.4 Restriction digestion of DNA

Plasmid DNA was digested with restriction endonucleases supplied with the restriction enzyme buffer under the conditions suggested by manufacturers. In the cases of double digestion, manufacturers' recommendations were used to find out the best buffer for the enzymes. In a typical digestion mixture, 5 units of enzyme were used for every 1  $\mu$ g of plasmid DNA. Restriction digestions were carried out, unless otherwise specified, in Eppendorf tubes and incubated for 1 hour at 37°C.

#### 2.5.5 Phosphorylation and Dephosphorylation

DNA fragments and synthetic oligonucleotides sometimes need to be phosphorylated before ligation or working as primers. Phosphorylation was catalyzed with T4 polynucleotide kinase in the presence of ATP. The buffer and the conditions were provided by the manufacturers. Dephosphorylation of DNA fragments was carried out with calf intestinal phosphatase under the conditions specified by the manufacturers. The reactions were heated at 70°C or extracted with phenol/chloroform to inactivate the enzymes.

#### 2.5.6 Blunting cohesive ends

3' recessed ends of DNA fragments were filled in using

Klenow fragment of *E. coli* DNA polymerase. The conditions were according to the protocol suggested by the manufacturers. 1 unit of Klenow was added per  $\mu\text{g}$  DNA in a reaction buffer supplemented with  $33 \mu\text{M}$  of each deoxynucleotide triphosphate.

3' overhang ends were blunted by removing the overhangs using T4 DNA polymerase under the conditions suggested by the manufacturers. 1-5 units of enzyme was used per  $\mu\text{g}$  of DNA. The reaction was carried out at  $12^\circ\text{C}$  for 20 minutes.

#### 2.5.7 Isolation and recovery of DNA fragments

DNA fragments were analyzed and isolated by electrophoresis in 0.8% agarose gels in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2.5 mM EDTA) containing 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide. The gels were run in the same buffer at about 15 volts/cm using a submarine gel apparatus. DNA fragments were observed and pictured under short wavelength ultraviolet light (Foto/PrepI, Fotodyne, Inc.).

There were three ways of recovering desired DNA fragments from agarose gels.

##### 1) Qiaex kit

The DNA fragment of interest was sliced from agarose gel and minced with a clean scalpel. 300  $\mu\text{l}$  of QX1 was added into every 100 mg of gel. After being well suspended by vortexing, 10  $\mu\text{l}$  of Qiaex beads were added for every 5  $\mu\text{g}$  of DNA. The gel and beads was mixed well in an Eppendorf tube and

incubated at 50°C for 10 minutes (mix every 2 minutes to keep Qiaex in suspension). The beads were collected by centrifugation and washed twice with 500  $\mu$ l of QX2 then twice with QX3. After removal of the supernatant, the pellet was air-dried for 15 minutes. The DNA was eluted with 20  $\mu$ l of TE (pH 8.0).

## 2) SpinBind™

Three gel volumes of binding solution (7.2 sodium iodide, 0.1 M sodium phosphate, pH 6.0) were added to the agarose gel slice and incubated at 60°C until the gel dissolved. The sample was applied onto a SpinBind unit (FMC Corp.) fitted in an Eppendorf tube. After washing once with NaI wash solution then twice with ethanol wash solution, the DNA was eluted with TE (pH 8.0).

## 3) Electroelution

The gel slice containing desired DNA fragments were placed in the horseshoe shaped slots of the electroelutor (model UEA, International Biotechnologies, Inc.) filled with just enough electro-elution buffer to cover the gel slice. High salt cushion (75  $\mu$ l of 7.5 M  $\text{NH}_4\text{OAc}$ ) was placed into the V shaped channels. DNA fragments were eluted by running the unit at 90 Volts for 45 to 90 minutes depending on the length of the fragment. The salt cushion was then removed and placed into an Eppendorf tube. The DNA fragments were precipitated with ethanol and dissolved into TE buffer.

### 2.5.8 Ligation of DNA fragments

T4 DNA ligase was used for the ligation of DNA fragments. However, the conditions for ligation of cohesive and blunt ends were different. For most cohesive ends ligation, 1  $\mu$ g of vector and a molar excess of insert were incubated 4 to 16 hours at 16°C with 10 units of T4 DNA ligase under buffer condition recommended by the manufacturer. Ligation of blunt ended DNA fragment required at least 50 times as much enzyme to achieve the same extent of ligation as cohesive end DNA fragments.

## 2.6 *E. coli* expression systems

### 2.6.1 Protein A fusion protein system

The bacterial protein A expression vector, pRIT2T has a thermoinducible  $\lambda$  right promoter ( $P_R$ ) controlled *Staphylococcus* protein A gene, a pBR322 replication origin (ori) and an ampicillin resistant gene ( $Amp^r$ ). Plasmid replication occurs in *E. coli* at 30°C while the protein A gene is repressed due to the presence of the temperature sensitive bacterial repressor, cI857, which is constitutively produced in N4830-1 bacterium strain. The expression of protein A gene can be induced by shifting the temperature to 42°C to inactivate the repressor. Because the protein A has 5 IgG binding domains, it can be easily purified by affinity chromatography using immunoglobulin G coupled Sepharose 4B (Pharmacia) column.

The gene of interest was cloned into the pRIT2T multiple cloning site located downstream from the protein A gene so that it could be expressed as a protein A fusion protein when the gene was placed in frame. The construction was transformed into the *E. coli* N4830-1 competent cells. The bacteria harbouring pRIT2T plasmid were grown in 2YT medium containing 50  $\mu\text{g/ml}$  ampicillin at 30°C to an O.D.<sub>600</sub> reading 0.6-0.8. The production of protein A was then induced by the addition of equal volume of 2YT medium pre-warmed to 60°C because of the thermo-induction of P<sub>R</sub> promoter through the temperature shifting to 42°C. The cells were incubated at 30°C for an additional 2 hours then harvested by centrifugation using Beckman GSA rotor at 5,000 rpm for 10 min at 4°C. The bacteria were washed and resuspended into the lysis buffer (13% sucrose, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10 mM EDTA, 0.1% NP40, 1mM PMSF and 0.25 mg/ml freshly made lysozyme). After incubation on ice for 30 minutes, the lysed bacteria were centrifuged at 45,000 rpm for 1.5 hours at 4°C using a Beckman Ti-50.2 rotor.

Affinity chromatography was performed with the Bio-Rad Econo pumping system at 4°C. The IgG sepharose 6FF (Pharmacia) was packed in a Bio-Rad column (10 mm X 100 mm) and equilibrated with TST (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20). The cell supernatant was applied to the column and washed with 10 bed volumes of TST, then 2 bed volumes of 5 mM NH<sub>4</sub>Ac, pH 5.0. The sample was eluted with 0.5

M HAc, pH 3.4 and collected by a fraction collector. After protein analysis using Bio-Rad protein dye, the protein containing fractions were immediately exchanged into buffer D (20 mM HEPES, pH 7.9, 0.1 M KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol) with a PD-10 column (Pharmacia). The purity of the protein was examined by SDS-polyacrylamide gel electrophoresis.

#### 2.6.2 Glutathione S-Transferase (GST) fusion protein system

The *E. coli* expression vectors, pGEX contain a *tac* promoter for IPTG inducible high-level expression, an internal *lac I<sup>q</sup>* gene for use in any *E. coli* strains and a thrombin or Factor Xa protease recognition site for cleaving the desired protein from the fusion products (Smith and Johnson, 1988). The genes or gene fragments of interest were cloned into the multiple cloning site of pGEX and expressed as fusions with *Schistosoma japonicum* glutathione S Transferase (GST). The fusion proteins were easily purified from bacterial lysates by affinity chromatography using glutathione Sepharose 4B (Pharmacia).

Bacterial cells harbouring appropriate pGEX plasmid were inoculated into 10 ml of 2YT medium containing 50 µg/ml ampicillin and grown at 37°C overnight. The overnight culture was diluted into fresh 2YT medium containing 50 µg/ml ampicillin with the 1:100 ratio and grown to an OD of 0.6. The

expression of the GST was induced by the addition of IPTG to a final concentration of 0.3 mM. After an additional 2 hours of incubation, the cells were pelleted by centrifugation, washed with PBS and resuspended into PBS containing 1% Triton X-100. A probe sonicator was used to lyse the cells with 3 ten second pauses every 30 seconds. The cell lysate was spun at 15,000 rpm for 20 minutes. The supernatant was applied on the glutathione Sepharose column pre-equilibrated with PBS + 1% Triton X-100. The column was washed with PBS until no protein was detected from the flow through. The bound material was eluted with elution buffer (5 mM glutathione in 50 mM Tris-HCl, pH 8.0) and collected by a fraction collector. Protein concentration was examined by Bio-Rad assay and the protein containing fractions were exchanged into buffer D using a F-10 column.

### 2.6.3 Maltose Binding Protein (MBP) fusion system

The *E. coli* expression vector pMAL-c2 (New England Biolabs), was also used to express Vmw65 and its mutants as MBP fusion proteins. It contained a strong *tac* promoter, which controlled a *malE* gene and was inducible by IPTG. The cloned gene was inserted into the multiple cloning site, which interrupted the fused *lacZ* gene from the *malE* gene (Guan et al., 1987). Therefore, the pMAL-c2 provided a good system for easy screening (Blue and white), high-level induction by IPTG and one-step purification using amylose affinity resin.

The bacterial cells transformed with pMAL plasmids were grown in 50 ml 2YT media containing 50  $\mu$ g/ml ampicillin at 37°C overnight. The overnight cultures were transferred into 500 ml of fresh medium and grown until the OD reached about 0.5. A final concentration of 0.3 mM IPTG was added to the cultures to induce the expression of malE gene. The cells were harvested by centrifugation and lysed by probe sonication as described above. The MBP and the fusion proteins were purified with amylose resins as described by the manufacturer using Bio-Rad Econo pumping system at 4°C. The PD-10 column was used to remove maltose and exchange the protein into buffer D. The protein amount was determined by the Bio-Rad assay and the protein purity was examined by SDS-PAGE electrophoresis.

## 2.7 Tissue culture

### 2.7.1 Maintenance of mammalian cell lines

Vero cells were maintained in Dulbecco modified essential medium (DME) supplemented with 10 % calf serum, 1% glutamine, 1% penicillin/streptomycin and incubated at 37°C in 5% carbon dioxide incubator on 10 cm dishes. Hela cells were grown in Joklik medium supplemented with 5% fetal calf serum, 2% glutamine, 1% penicillin/streptomycin, 1X vitamins and 1% non-essential amino acids. Upon reaching confluence, the cells were split at a 1 to 10 ratio by rinsing with phosphate buffered saline (PBS), trypsinizing and diluting into fresh



medium.

### 2.7.2 Growth of Hela cells in suspension

Hela cells were grown in suspension in a sterilized spinner flask containing appropriate amount of Joklik medium supplemented with 5% fetal calf serum, 2% glutamine, 1% penicillin/streptomycin, 1X vitamins and 1% non-essential amino acids. The cells were kept at 37°C in 5% carbon dioxide with stirring and maintained the density between  $2.0 \times 10^5$  and  $5.0 \times 10^5$  cells/ml by subculturing every 2-3 days as required.

### 2.7.3 Viral infections and plaque assays

Vero cells were grown on 10 cm dishes to confluence, then washed 3 times with PBS. The virus aliquot was added to the cells in 3 ml of fresh medium. After incubation at 37°C for 2 hours, the virus particle were absorbed onto the cells. 10 ml of medium was added to the incubation to allow the infection to proceed for the desired time.

Plaque assays were performed with 2 six well trays grown with confluent vero cells. Serial dilution ( $10^2$  to  $10^9$ ) of virus was prepared in 1 ml of DME- (DME without serum) because the presence of serum inhibited the adsorption of virus onto the cells. After removing the media from the wells of the trays, 0.2 ml of each virus dilution was added along with the control containing no virus. The cells were incubated at 37°C for 2 hours with shakes every 15 minutes. 3 ml of DME

containing 0.05% HGG (human gamma globulin) was added into the infection and allowed to proceed for 2 to 3 days. The virus titre (PFU/ml) was examined by counting the plaques on each well.

#### 2.7.4 Preparation of whole cell extract from Hela cells

Whole cell extract was prepared from suspension culture of Hela cells as described by Manley *et al.* (1983). Typically, 1 liter of Hela cell suspension at the density of  $5-6 \times 10^5$  would yield 20 mg of protein. The whole procedure was carried out at 4°C. Upon reaching the density of  $4-6 \times 10^5$  cell/ml, the Hela cell suspension was harvested by centrifugation with a GSA rotor at 1,000 rpm for 10 minutes. The cells were washed twice with PBS and determined the PCV (pellet containing volume), then resuspended in 4 PCV of buffer A (10 mM Tris, pH 7.9, 1 mM EDTA, 5 mM DTT). After incubation on ice for 20 minutes (allowed the cell to swell), the cells were lysed by homogenizing using a dounce pestale B with 8 strokes. 4 PCV of buffer B (50 mM Tris, pH 7.9, 10 mM  $MgCl_2$ , 2 mM DTT, 25% sucrose, 50% glycerol) was added to the cell lysate and mixed well gently. With continued gentle stirring, 1 PCV of saturated ammonium sulphate was added dropwise. The mixture was stirred gently for 30 minutes then spun at 45,000 rpm for 3 hours using a Beckman sw 50.2 rotor. The supernatant was transferred to another clean tube without disturbing the pellet. The protein and nucleic acid were

precipitated by adding solid ammonium sulphate to the final concentration of 0.33 g/ml solution. After ammonium sulphate is dissolved, 1 N NaOH (0.1 ml/10 g solid ammonium sulphate) was added with stirring and continued to stir for 30 minutes. The precipitate was collected by centrifugation at 15,000 rpm for 20 minutes using a ss34 rotor. The pellet was resuspended in 5% supernatant volumes of buffer C (25 mM HEPES, pH 7.9, 10 mM MgCl<sub>2</sub>, 0.1 M KCl, 0.5 mM EDTA, 2 mM DTT, 17% glycerol) and dialysed against 2 changes of 50-100 volumes of buffer C for 8-12 hours. The dialysate was centrifuged at 10,000 rpm for 10 minutes. The supernatant was ready for the analysis or quickly frozen in liquid nitrogen and stored at -80°C.

#### 2.7.5 Preparation of nuclear extract from HeLa cells

Nuclear extract was prepared from HeLa cell suspension culture using the method described by Dignam *et al.* (1983) with some modifications. The whole procedure was carried out at 4°C. HeLa cell suspension with the density of 4-6 X 10<sup>5</sup> was pelleted at 1,500 rpm for 15 minutes using a Beckman GSA rotor. The cells were washed twice with PBS and determined the PCV. The cells were then resuspended in 5 PCV of buffer A (10 mM HEPES, pH7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT) and left on ice for 10 minutes to allow the cells to swell. The cells were centrifuged at 2,000 rpm for 10 minutes and resuspended in 2 PCV of buffer A + 0.5% NP40 with intermittently mixing during a 10 minute period. The lysate was spun at 2,000 rpm

for 10 minutes to separate the nuclei. The supernatant was removed for the preparation of S-100 post-nuclear extract and the pellet was again spun at 25,000 rpm for 20 minutes. The pellet was resuspended in 3 ml of buffer C/ $10^9$  cells (buffer C: 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 1.5 mM MgCl<sub>2</sub>) and dounced 10 strokes with pestle B to break nuclear membrane. The extract was stirred gently for 30 minutes and then centrifuged at 13,000 rpm for 30 minutes using a Beckman SA600 rotor. The supernatant was dialysed 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, 0.5 mM DTT) for 12 hours and then centrifuged again at 13,000 rpm for 30 minutes to collect the nuclear extract.

#### 2.7.6 Preparation of viral protein extract

Ten 15 cm plates of confluent vero cells were infected with HSV-1 strain 17 at an moi of 20. The cells were incubated at 37°C for 24 hours and harvested by scraping with a rubber policeman. After spinning at 1,000 rpm for 5 minutes at 4°C, the supernatant was removed and stored at -80°C. The pellet was resuspended in 5 ml of supernatant, freezeed and thawed three times, and sonicated for three 10 second periods. The cell lysate was spun at 2,000 rpm for 10 minutes at 4°C. The supernatant was added to the original supernatant. Together, the supernatant was centrifuged at 16,000 rpm for 2 hours at 4°C using a Beckman Type 19-HP6 rotor. The pellet was

resuspended in PBS containing 0.5% calf serum and then centrifuged through 2 ml of 10% w/v PBS sucrose cushion at 40,000 rpm for 1 hour at 4°C using a Beckman SW 41Ti rotor. The pellet was resuspended in 1.5 ml of a solution containing 10 mM Tris, pH7.5, 50 mM NaCl, 1 mM EDTA. NP40 was added to the mixture to a final concentration of 0.03%. The mixture was placed on ice for an hour and centrifuged at 30,000 rpm for 1 hour at 4°C using a Beckman SW 50.1 rotor. Glycerol was added in the supernatant to a final concentration of 10%. The extract was divided into 200  $\mu$ l aliquots and stored at -80°C.

## 2.8 Analysis of protein-protein interaction using micro affinity assay

### 2.8.1 Coupling of MBP and fusion proteins to the amylose resin

Amylose resin (New England Bio-Labs) was pre-treated with the following procedure: two washes with 10 volumes of column buffer (20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 1 mM  $\text{NaN}_3$ ); one wash with column buffer (CB) + 0.25% Tween 20; two washes with buffer D (Dignam et al., 1983). For each 100  $\mu$ l of settled beads, 250  $\mu$ g of MBP or Vmw65 fusion proteins were added. After incubating at 4°C overnight with rotation, the beads were collected with low speed centrifugation and washed three times with each of the 10 volumes of buffer D, 10 volumes of buffer D containing 100  $\mu$ g/ml BSA, and 10 volumes of buffer D containing 100  $\mu$ g/ml BSA

and 0.05% NP40.

### **2.8.2 Incubation with VCAF-1**

Twenty  $\mu$ l of settled beads with affinity ligands were incubated with 20  $\mu$ l of affinity purified VCAF-1 for 2 hours at 4°C with continuous rotation. The mixture was spun down with low speed centrifugation and the supernatant containing non-bound material was collected for the mobility shift assay. The beads were washed three times with 10 volumes of buffer D containing 100  $\mu$ g/ml BSA and 0.05% NP-40 followed by three times with 10 volumes of buffer D. The material bound onto the beads was eluted with 20  $\mu$ l of buffer D containing 0.6 M KCl. Mobility shift assay was performed with the supernatant and eluent from above micro-affinity assay to define VCAF-1 in the incubation mixture containing PA-POU, Vmw65 and DNA probe to examine the VCAF-1 activity.

## **2.9 Oligo-nucleotide directed mutagenesis**

Oligo-nucleotide directed mutagenesis is widely used for studying the structure and function of proteins by introducing a point mutation at specific sites in the primary sequences.

### **2.9.1 Preparation of the uracil containing single stranded DNA template**

The full length Vmw65 was subcloned into the BamHI

site of M13/19RF (replicate form) and the recombinant DNA was transformed into a *dut-*, *ung-* double mutant bacterial strain *E. coli* CJ-236. In this strain, the nascent DNA carries uracil instead of thymine as the result of inactivating dUTPase and uracil N-glycosylase. The transformed bacteria were plated on the top agar of LB medium with the overnight cultured CJ-236 cells. The next day, well isolated plaques were observed on the plate. To grow the uracil containing template for mutagenesis, a single colony of CJ-236 bacterium was inoculated into 20 ml of fresh LB medium and cultured overnight at 37°C. The following morning, 1 ml of the above culture was inoculated into 50 ml of 2YT medium containing 0.25 µg/ml of uridine and 15 µg/ml of chloramphenicol and incubated at 37°C for 2 hours. The incubation was infected with a single plaque of M13 phage from CJ-236 top agar and incubated for another 6 hours. After spinning down the bacterial cells, the supernatant was precipitated with 1/4 volume of 2.5 M NaCl+20% polyethylene glycol (PEG) 8000 and the phage particles were collected by centrifugation. The phage DNA containing uracil was extracted with phenol and chloroform and precipitated with ethanol. Titering phage was done to determine whether the single stranded DNA contained uracil. The results demonstrated that the ratio of infection on wild type (MV1190) and *dut-*, *ung-* (CJ-236) bacteria by uracil containing phage was over  $1:10^{10}$ .

### 2.9.2 Synthesis of the complementary mutant strand

The following oligo-nucleotides were designed to substitute selected charged amino acid (arginine, cystine and lysine) residues with alanine at various positions within the two regions of Vmw65, and used as primers for the *in vitro* synthesis of the mutagenic strand.

In region 1:

R155A 5'-GCG CTC GCT TTC TTC C  
R162A 5'-C GCC GAG CTA GCG GCG CGG G  
R164A 5'-G CAT CGG GCG GCG GAG GAG ACC  
R169A 5'-G GAG AGC TAT GCA ACC GTG TTG G  
C176A 5'-GCC AAC TTC GCC TCG GCC GTG

In region 2:

R360A 5'-G GCG GTC ATG GCG GAA CAC GCG  
R366A 5'-C GCG TAC AGC GCT GCG CGT ACG  
R368A 5'-C AGC CGC GCG GCT ACT AAA AAT TAC  
K370A 5'-GCG CGT ACG GCA AAC AAT TAC G

The altered amino acid codons are underlined.

The above synthetic oligonucleotides were phosphorylated with T4 polynucleotide kinase before priming. The annealing mixture that contained 200 ng of uracil containing single stranded DNA, 9 pmole of mutagenic oligonucleotide, 1x annealing buffer (20 mM Tris-HCl, pH 7.4, 2 mM MgCl<sub>2</sub>, 50 mM NaCl) was prepared and incubated at 70°C for 2 min then cooled at a rate of 1°C/min to 30°C. T7 DNA



polymerase was used to synthesize the complementary strand. The reaction also contains each of the 4 dNTPs, ATP and T4 DNA ligase. As a result, the synthesized M13/RF is the hybrid of the uracil containing template strand and the wild type complementary mutagenic strand.

### **2.9.3 Transformation of the hybrid M13/RF into MV1190 competent cells**

After synthesis, the M13/RF containing the point mutation on its complementary strand was transformed into a wild type bacterial strain (MV1190) to select for the mutation. Due to the presence of the uracil N-glycosylase, the uracil containing strand is highly inactivated and cannot be replicated, leaving the mutagenic strand to survive and replicate. Briefly, the synthesis mixture was added into MV1190 competent cells and incubated on ice for 45 min and heat shocked at 42°C. The transformation mixture was then plated on LB top agar with overnight cultured MV1190 cells. The well isolated plaques were observed on the plate on the following day.

### **2.9.4 Screening of the mutations by DNA sequencing**

Because the frequencies in obtaining a mutant with this technology are relatively high (usually 50% to 75% or higher), it allows the mutants to be directly identified by sequencing analysis without phenotype screening. Single-

stranded DNA of M13 phage is prepared from a well isolated plaque on the plate infected with the mutant containing transformation. Briefly, 1 ml of overnight cultured MV1190 cells was diluted into 50 ml of fresh LB medium and 5 ml of diluted bacteria were dispensed into 15 ml culture tubes. Each tube was inoculated with a separate plaque using a sterile loop and incubated at 37°C for 6 hours. After removing the cells by centrifugation, the culture supernatant was precipitated with PEG/NaCl. The phage particles were collected and resuspended in Tris/EDTA solution. The phage DNA was extracted with phenol/chloroform and precipitated with ethanol.

Chain-termination (dideoxy) sequencing technology was used, with the reagents provided by a Sequenase version 2 kit (United States Biochemicals). The manufacturer's suggested procedure was followed, and the annealing temperature for the sequencing primer was 65°C. The sample was run on an 8% acrylamide sequencing gel containing 8 M urea at 55 watts of constant power. The primer for sequencing Vmw65 region 1 is 5'-GCG TAC GTC CCC GAA CGC ACC, which is positioned at amino acid residues 118-124, and the primer for region 2 is 5'-C CTG CAT GGC AAC CAG GCC CGC G which is positioned at amino acid residues 324-332. The results showed that 75% of the plaques contain the mutations introduced by mutagenic oligonucleotides.

## 2.10 Transactivation of HSV ICP4 gene promoter and chloramphenicol acetyltransferase (CAT) assay

### 2.10.1 Subcloning of Vmw65 and point mutants into eukaryotic expression vector pEVRF

Eukaryotic expression vector pEVRF was used to express and analyze the mutant proteins of Vmw65. pEVRF has a very high efficiency of expressing mutant cDNAs in eukaryotic cells (Matthias et al., 1988). It contains a pSP65 bacterial plasmid backbone, a human CMV promoter, mRNA splicing and polyadenylation sites, a translation initiation site of HSV thymidine kinase gene and an SV40 replication of origin. Therefore it can shuttle between bacteria and eukaryotic cells for the cloning and expression purposes. The full length Vmw65 was first cloned into BamHI site of pEVRF (by N. Shen). The subcloning of the mutant genes were performed by replacing the Sal fragment of the wild type gene with the mutant ones. After removing the Sal fragment, the plasmid was dephosphorylated with CIAP and separated by gel electrophoresis. The Sal fragment of the mutant genes were gel separated and purified with the Qiaex kit. The constructions were then transformed in DH-5 $\alpha$  and screened for proper orientations.

Plasmid p $\alpha$ 4CAT (constructed by Dr. Capone) is used for testing the *in vivo* transactivation of HSV immediate early gene by Vmw65. It contains a CAT reporter gene which is fused to an HSV ICP4 gene promoter, therefore, can be induced by Vmw65. When p $\alpha$ 4CAT is co-transfected with Vmw65 expression

vector pEVRF, the activation of the HSV IE gene (ICP4) promoter by Vmw65 can be measured by examination of the enzyme activity of CAT. Plasmids for transfection were prepared by Qiagene columns and quantified by a fluorometer.

#### 2.10.2 Transient transfection

Vero cells were used as host cells for the transfection of pEVRF and p $\alpha$ 4CAT plasmids and maintained in complete DME medium. Transfections were carried out using the calcium phosphate coprecipitation method previously described (Graham and Van der Eb, 1973). The day before transfection, cells were seeded on 6 cm dishes at the density of 10,000 cell/ml and incubated at 37°C in a carbon dioxide incubator until cells grew to 50-80% confluent. The transfection mixture contained the desired amount of plasmid DNA and the total DNA was normalized at 20  $\mu$ g/dish with sonicated salmon sperm DNA (Pharmacia). 31  $\mu$ l of 2 M calcium phosphate was added to the mixture. The final volume of the mixture was adjusted to 0.25 ml by the addition of water. To the mixture, 0.25 ml of 2X HBS (50 mM HEPES, 0.28 M sodium chloride, 1.5 mM sodium phosphate, adjusted to pH 7.12 with sodium hydroxide) was slowly added by dropping over a 30 second time span and mixed well by vortexing. After incubating at room temperature for 20 minutes, a fine precipitate was observed and the mixture was added into the plate of cells containing 5 ml of fresh medium. The plates were incubated at 37°C in 5% carbon dioxide for 4

hours. The cells were shocked with DME- medium containing 10% dimethyl sulfoxide (DMSO), washed 3 times with DME- medium and incubated in complete DME medium for 40 to 48 hours at 37°C in 5% carbon dioxide.

### 2.10.3 Harvesting the transfected cells

The cells ready for chloramphenicol acetyltransferase assay were harvested by washing 3 times with PBS, incubating with 1 ml TEN buffer (40 mM Tris-HCl, pH 7.4, 1.5 M sodium chloride, 1M EDTA) for 5 minutes on ice, then scraped with a rubber policeman and transferred into Eppendorf tubes kept on ice. The cell suspension was pelleted by spinning for 2 minutes at 4°C with a microfuge. The cells were resuspended in 100  $\mu$ l ice cold CAT buffer (250 mM Tris, pH 7.8, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.5% NP40) by vortexing. The tubes were sonicated for 3 X 10 seconds using a water bath sonicator (Branson 1200) and spun for 5 minutes at 4°C with a microfuge. The supernatant containing the cell extract was transferred to a clean Eppendorf tube for the CAT assay.

### 2.10.4 Chloramphenicol acetyltransferase (CAT) assay

CAT assay was performed using the method to measure the diffusion of reaction products into scintillation fluid (Sambrook et.al, 1989). The principle of this method is that the CAT gene product will catalyze the transfer of radio

labelled acetyl group to chloramphenicol to produce acetylchloramphenicol, which diffuses from aqueous reaction directly into an organic scintillation fluid. It is then be quantitated by counting over selected time intervals (Neumann et. al, 1987). 50  $\mu$ l of cell extract decried above was used for CAT assay and heated at 70°C for 15 minutes. The supernatant was separated by centrifugation for 10 minutes at 4°C and placed into a mini vase, in which 200  $\mu$ l of pre-assay mixture (12.5  $\mu$ l Tris, pH 7.8, 50  $\mu$ l 5 mM chlcramphenicol, 0.1  $\mu$ Ci  $^3$ H-acetyl Coenzyme A (10 Ci/mmole) and water to 200  $\mu$ l) was added, immediately followed by the addition of Econoflour scintillation fluid. The samples were immediately placed in a scintillation counter to record the cpm (count per minute) and counted for 10 cycles over 10-15 minutes intervals. The relative CAT activity was calculated by the slope of the cpm versus reaction time.

## CHAPTER 3

### IDENTIFICATION AND PURIFICATION OF THE CELLULAR FACTOR VCAF-1

#### 3.1 Background

A fundamental aspect of regulating transcription in eukaryotic cells involves the selective recruitment of transactivators and other protein factors to assemble into functional complexes on specific DNA elements. The activation of HSV-1 immediate early genes has served as a model for understanding the mechanism of regulatory assembly of multi-component complexes and control of gene expression patterns (Goding and O'Hare, 1989; Thompson and McKnight, 1992). Upon lytic infection, the genes of HSV are expressed in a regulated temporal fashion, which is combinatorially controlled by both viral and cellular factors through distinct protein-protein and protein-DNA interactions.

Early findings showed that Vmw65 had no efficient DNA binding activity to either the TAATGARAT consensus or general DNA sequences (Marsden et al., 1987). In order to activate HSV IE genes, Vmw65 must be assembled into a multi-component complex with other cellular factor(s) (Kristie and Roizman, 1987; McKnight et al., 1987). The first important finding to

support this theory was that, in many HSV IE gene promoters, the TAATGARAT elements overlapped with an octamer sequence motif (ATGCAAAT) (Prujin et al., 1986; O'Hare and Goding, 1988; apRhys et al., 1989). An ubiquitous octamer binding protein, Oct-1 (also referred to as OTF-1, TRF, and NFIII) that binds to the ATGCAAAT element, was subsequently identified as a cellular factor necessary for the formation of a DNA-binding complex with Vmw65 (Gerster and Roeder, 1988; O'Hare and Goding, 1988; O'Hare et al., 1988). The study by Gerster and Roeder (1988) also suggested that other cellular factor(s), distinct from Oct-1, was required for complex assembly because Vmw65 and Oct-1 alone were not sufficient to form a stable complex at the TAATGARAT element.

The fact that affinity purified Oct-1 from HeLa cell extract was unable to form the DNA-binding complex with Vmw65 (Gerster and Roeder, 1988), suggested that this additional factor did not directly interact with Oct-1. If it did, the complex was very unstable. Another consideration was there was no evidence to show whether Oct-1 directly interacts with Vmw65 in the absence of DNA although it is necessary for complex formation. Therefore, it was likely that this additional factor might directly interact with Vmw65. To answer these immediate questions regarding the assembly of the complex, we set out to fractionate HeLa cell extract using affinity chromatography with Vmw65-coupled columns.



Previous experiments carried out in this laboratory had demonstrated that Vmw65 expressed in *E. coli* as a fusion protein with *Staphylococcus aureus* protein A had the same properties in mobility shift assay as virion Vmw65, and did the Sal fragment (residues 5 to 411), which was constructed by removing the coding region for the 79 amino acid AAD from the carboxyl terminal (Werstuck and Capone, 1989b). Because of its ability to generate the Vmw65-induced complex at the TAATGARAT element, the *E. coli* expressed protein A fusion Sal fragment was utilized as ligand in affinity chromatography to separate cellular factors that potentially bound to Vmw65 independently. Because the Sal fragment did not encode the acidic activation domain, the artificial affects of highly negatively charged amino acids in the full-length Vmw65 would be minimized.

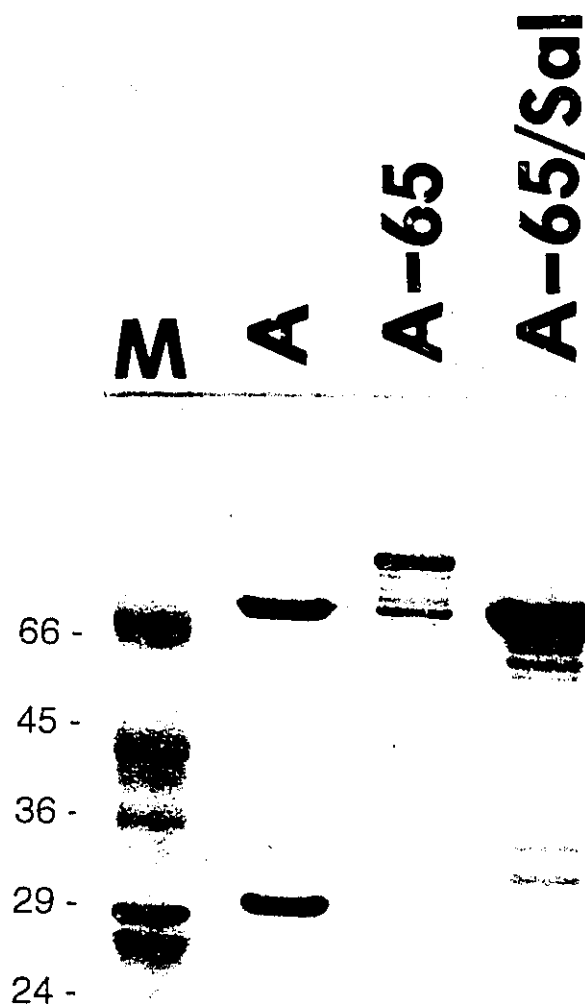
### 3.2 Materials and Methods

The materials and methods directly related to the identification of cellular factor required for Vmw65-induced complex assembly are put in this section.

#### 3.2.1 Preparation of affinity columns

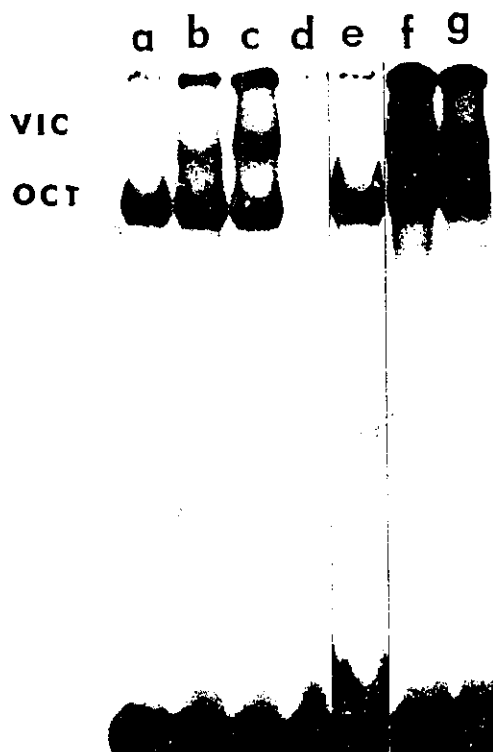
Vmw65 and its Sal fragment were cloned into the *E. coli* expression vector pRIT2T (Werstuck and Capone, 1989b). The expression and purification of *S. aureus* protein A fusion proteins were carried out as described in Chapter 2. The

analysis of fusion proteins by SDS-polyacrylamide gel electrophoresis found that each purified protein generated a major band on the gel and these bands corresponded to the expected molecular weights of protein A and protein A fusions (Figure 3.2.1). Besides the specific bands, a band that co-migrated with Protein A-Sal was also present in all the preparations. This band was believed to be non-specific since it was also present in bacterial lysates without plasmid transformation (data not shown). More importantly, the mobility shift assay showed that fusion proteins of PA-Vmw65 and PA-Sal were able to generate VIC with HeLa cell extract while protein A was not capable of forming VIC (Figure 3.2.2).



**Figure 3.2.1** Purification of protein A-Vmw65 fusion proteins from *E. coli*

DNA sequences corresponding to full-length Vmw65 and Sal fragment (4-411) were cloned into the protein A expression vector pRIT2T. Fusion proteins were purified from induced culture by affinity chromatography on IgG Sepharose. Shown is the Coomassie blue-stained profile of the purified proteins. M; molecular weight marker. A; protein A. A-65; full-length protein A-Vmw65. A-65/Sal; protein A-Sal fragment.



**Figure 3.2.2** Protein A-fused Vmw65 and Sal fragment were able to generate VIC with HeLa cell extract

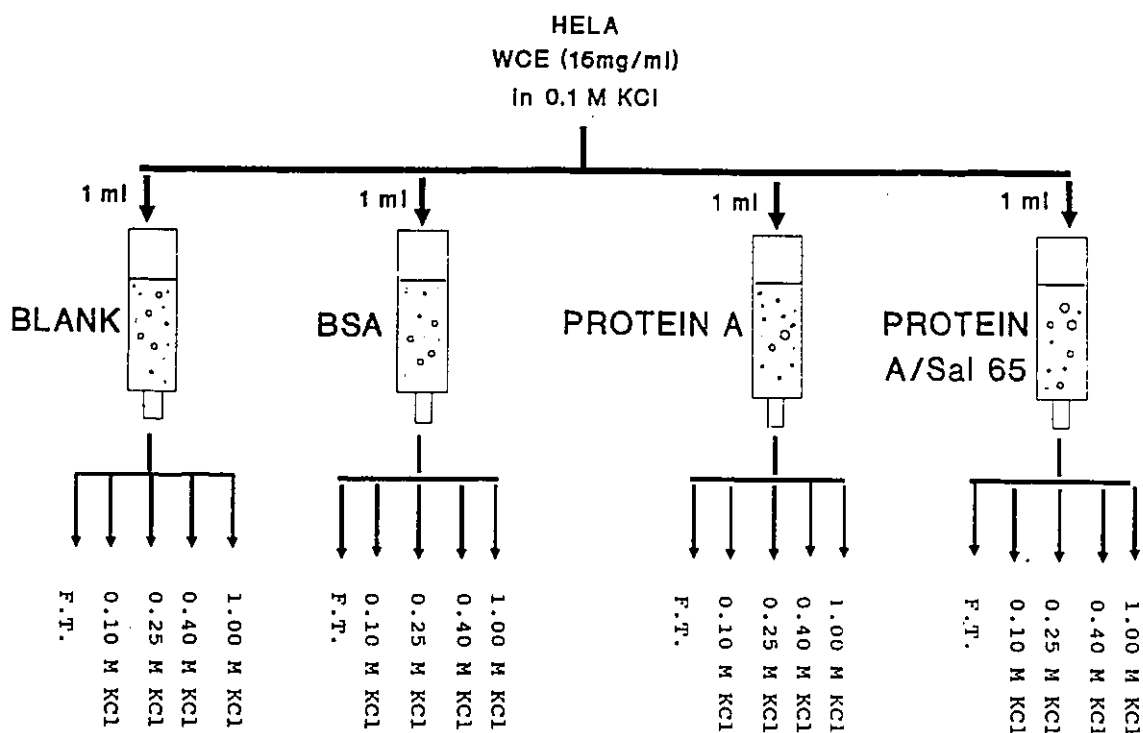
Twenty  $\mu\text{g}$  of HeLa whole cell extract was incubated with a synthetic DNA probe containing the ICP0 promoter proximal ATGCTAATGARAT element in the absence (lane a) and presence of 0.05  $\mu\text{g}$  of Vmw65 from HSV extract (lane b). Lane c is the same as b but includes LP1, a monoclonal antibody against Vmw65 to observe a supershift. Lanes e to g were incubations of HeLa cell extract with 0.5  $\mu\text{g}$  of protein A, A-65 and A-Sal respectively. Incubation containing only Vmw65 from HSV can not form DNA binding complexes (lane d). Reactions were supplemented with 4  $\mu\text{g}$  of poly(dI-dC)/salmon sperm DNA (1:2) and 5  $\mu\text{g}$  of BSA and resolved on a 3.5% native polyacrylamide gel.

Cyanogen bromide-activated Sepharose 4B (Pharmacia) was chosen as the matrix to couple protein A fusion proteins. The beads were pretreated with 1 ml HCl according to the instructions of the manufacturer. Immediately before coupling, the gel was activated by washing once with coupling buffer (CB) (0.1 M NaHCO<sub>3</sub>, pH 8.3, 0.5 M NaCl). Five mg of purified protein fused Vmw65/Sal in 2.5 ml of CB was added to 1 ml of settled beads, and the mixture was gently rotated overnight at 4°C. The gel was collected by low speed centrifugation and the beads were resuspended in 5 volumes of 1 M glycine, pH 8.0 + 1 mM DTT and incubated for an additional 8 hours at 4°C with rotation in order to block the remaining reactive groups. The gel was collected by low speed centrifugation and washed sequentially with 10 volumes each of CB, 0.1 M acetate, pH 4.0 + 0.5 M NaCl, and 0.1 M Tris-HCl, pH 8.0 + 0.5 M NaCl. After repeating the above washing cycle twice, the gel was finally washed with the affinity column buffer (ACB) (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 50 mM KCl, 0.5 mM PMSF, 100 kU of apoprotein per ml, 10% glycerol), resuspended in appropriate amounts of ACB and stored at 4°C. As controls, protein A purified from pRIT2T and bovine serum albumin (BSA) were coupled to the CN-Br activated Sepharose beads in parallel under the identical conditions. The affinity gel that contained no immobilized ligand but in which reactive groups were neutralized with 1 M glycine (blank column) was used as an additional control. Protein coupling efficiency in all

cases was greater than 95%.

### 3.2.3 Affinity chromatography

Affinity chromatography was carried out at 4°C using 2 ml disposable columns (Bio-Rad) packed with 300  $\mu$ l of affinity gel or control gels. Columns were pre-washed sequentially with 10 column volumes of ACB, ACB containing 0.1 mg/ml BSA, and again ACB. Prior to application, the HeLa whole cell extract was diluted with an equal volume of ACB without KCl to reduce the salt concentration to 5 mM. 1 ml (20 to 25 mg of protein) of the extract was applied to Vmw65 and control columns at a flow rate of 2 ml/hour. The flow through from each column was reapplied twice and collected. The columns were washed sequentially with 10 column volumes of ACB, ACB containing 0.05% Tween 20, and finally ACB. The bound material was step eluted with 4 column volumes each of ACB containing 0.1, 0.25, 0.4, and 1 M KCl. This was followed by 4 column volumes of 0.2 M acetic acid, pH 3.4. The fractions were collected and exchanged into ACB with PD10 columns (Pharmacia). Centricon 10 microconcentrators (molecular weight cutoff 10,000 Da; Amicon Corp.) were used to concentrate the fractions to 200  $\mu$ l volume. Figure 3.2.3 shows the diagram of fractionation of HeLa whole cell extract by columns coupled with BSA, protein A and protein A fused Sal fragment, as well as the column with conjugating sites inactivated.



**Figure 3.2.3** Affinity columns were prepared by coupling purified proteins with CNBr-activated Sepharose (5 mg protein/ml of settled gel). Chromatography was carried out at 4°C using columns packed with 300  $\mu$ l of coupled gel. HeLa whole cell extract was chromatographed on each column in parallel under identical conditions. The flow through (FT) from each column was collected and bound material was step eluted with affinity column buffer (ACB) containing increasing amounts of KCl as indicated.

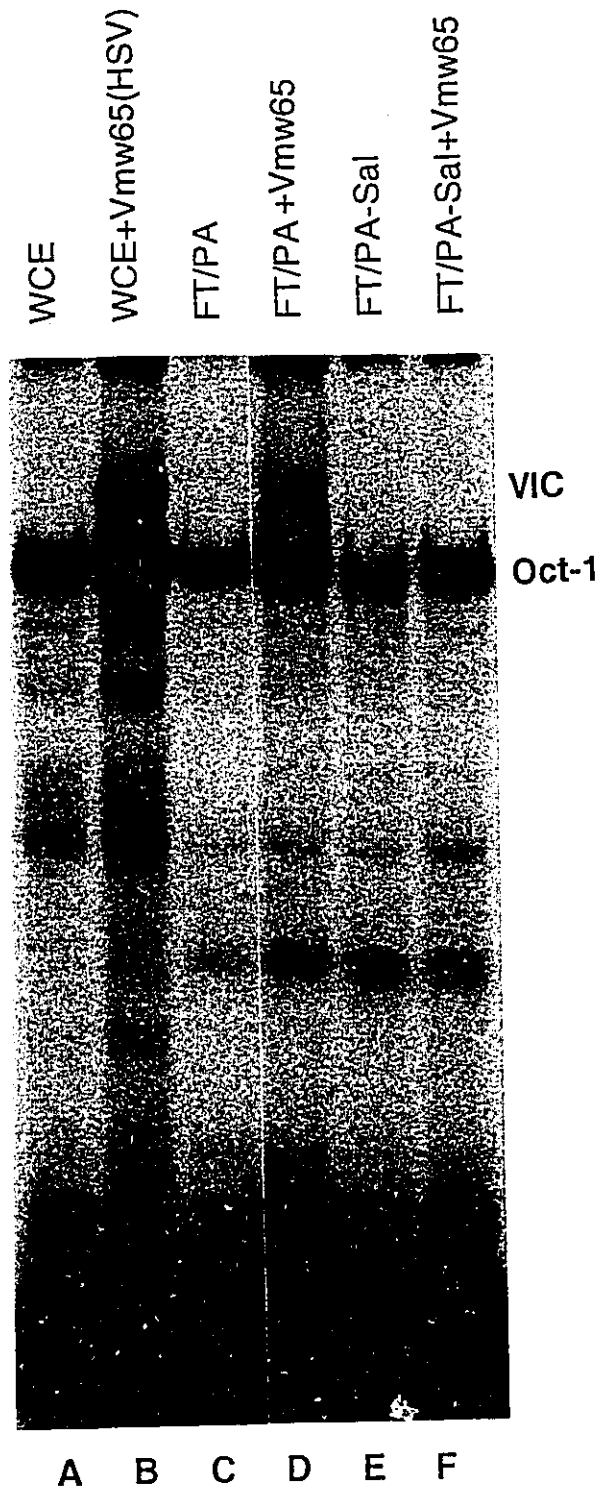
### 3.3 Results

#### 3.3.1 The excluded fraction from PA-Sal affinity column was defective in VIC assembly

It was predicted that cellular factors selectively bound to Vmw65 would be retained on a Vmw65-conjugated column, and therefore would be depleted from the HeLa cell excluded fraction. In other words, the excluded fraction from the Vmw65 affinity column would be unable to replace HeLa cell extract to generate the Vmw65 dependent complexes if the factor(s) bound to Vmw65 was necessary for complex formation. To test such a presumption, mobility shift assays were carried out with a radiolabelled oligonucleotide probe containing the promoter-proximal TAATGARAT element derived from the HSV-1 ICP0 gene. The mobility shift assays contained an appropriate amount of affinity chromatography fractions and 0.05  $\mu$ g of Vmw65 prepared from an NP40 extract of HSV-1 virion (Kristie and Roizman, 1987) per binding reaction as indicated.

In the pilot experiments to determine the conditions for affinity chromatography, we found that when HeLa whole cell extract was applied, the flow through from the column coupled with PA-Sal was unable to form VIC with Vmw65 but the flow through from protein A column was still capable of forming VIC (Figure 3.3.1). It was clearly indicated that some component important for VIC formation was depleted from the flow through of PA-Sal affinity column. However, this PA-Sal excluded fraction was still able to form the Oct-1-DNA





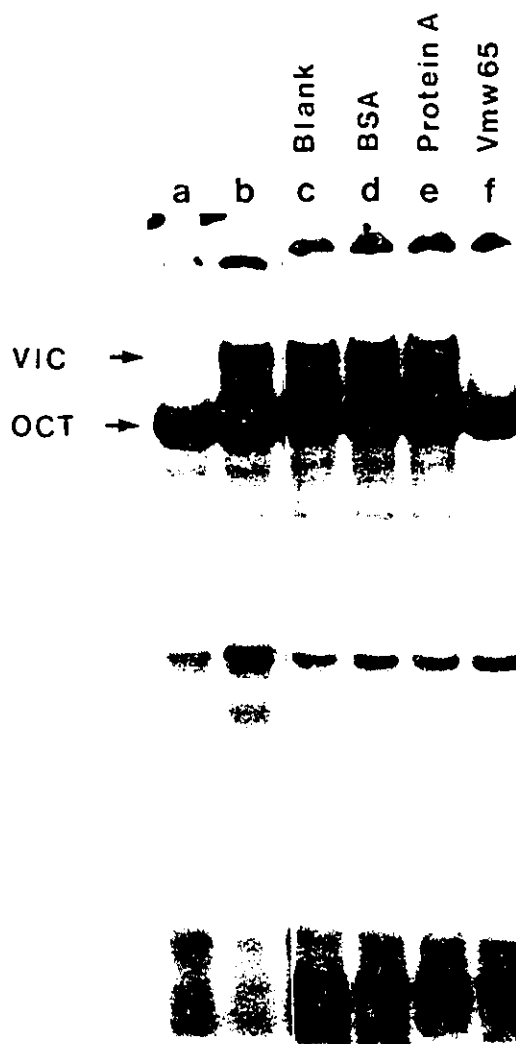
**Figure 3.3.1** Excluded fraction from PA-Sal coupled column was unable to form VIC with Vmw65

Excluded fractions from both PA (lane C and D) and PA-Sal (lanes E and F) coupled columns were incubated with the TAATGARAT probe in the absence (lanes C and E) or presence (lanes D and F) of Vmw65 derived from HSV extract. Lanes A and B contained 20  $\mu\text{g}$  of HeLa WCE alone (A) or with 0.05  $\mu\text{g}$  of Vmw65, respectively.

complex. Therefore, Oct-1 probably did not directly bind to Vmw65 in the absence of DNA.

To further examine if this component was specifically bound to Vmw65, HeLa whole cell extract was applied on the control columns containing Sepharose coupled with BSA, protein A, or with the coupling site inactivated, under the identical conditions.

Figure 3.3.2 shows the results of mobility shift assays using HeLa whole cell extract that was subjected to chromatography on the Vmw65 affinity column and control columns. Lanes a and b were controls showing the Oct-1-containing DNA-binding complex generated by incubation with whole cell extract prior to affinity chromatography. An additional shift was observed when Vmw65 was present in the incubation, hence demonstrating the formation of the Vmw65 induced complex (VIC). The presence of Vmw65 in the VIC can be confirmed by supershifting the complex with a monoclonal antibody against Vmw65 (LP-1) (data not shown). As demonstrated in Figure 3.3.2, the excluded fractions obtained following chromatography on the blank, BSA, or protein A columns were capable of generating both Oct-1 and VIC complexes in the presence of Vmw65. In contrast, the flow through from the Vmw65-coupled column, while capable of forming the Oct-1-DNA complex, was defective in its ability to form VIC in the presence of Vmw65.



**Figure 3.3.2** The cellular factor required for VIC formation specifically binds to the Vmw65 column

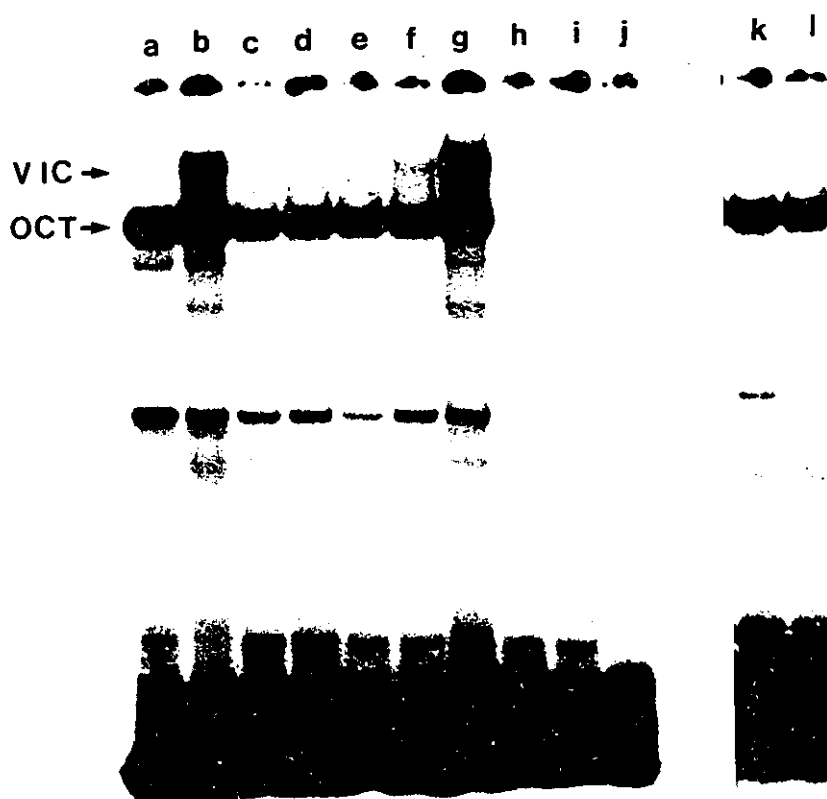
HeLa WCE was chromatographed on affinity columns containing no ligand (blank, lane c) or containing immobilized BSA, protein A, or PA-sal (lanes d to f, respectively). An equivalent amount (20  $\mu\text{g}$ ) of excluded fraction from each column was incubated with 0.05  $\mu\text{g}$  of Vmw65 and DNA probe. Lanes a and b are controls by incubating HeLa cell extract without chromatography in the absence and presence of Vmw65.

This experiment demonstrated two important features involved in the assembly of Vmw65 mediated multi-component DNA binding complex. First, another cellular factor, in addition to Oct-1, was required for VIC assembly, and this factor bound to Vmw65 independently. Second, the octamer binding protein, Oct-1, did not bind to Vmw65 in the absence of DNA, although it was required for complex formation.

### 3.3.2 The activity required for VIC formation was recovered from PA-Sal affinity column

The bound material on Vmw65-conjugated column was stepwise eluted by increasing the concentration of KCl (0.1, 0.25, 0.6 and 1.0 M). The eluted fractions were tested for their ability to reconstitute VIC formation with the depleted extract (Figure 3.3.3). The mobility shift assay demonstrated that fraction eluted with 0.1 and 0.25 M KCl had little effect on VIC formation when added back to the deficient extract (lanes d and e). The 0.6 M KCl fraction was capable of restoring some VIC formation ability to the defective extract (lane f), while the 1 M KCl fraction was able to significantly reconstitute VIC formation (lane g).

Mobility shift assays by incubating the 1 M KCl eluent with the DNA probe in the absence of protein extract had also demonstrated that the 1.0 M KCl fraction contained no specific DNA binding activity (lane h). In addition, no DNA binding



**Figure 3.3.3 Reconstitution of VIC with the salt eluted fractions**

The excluded fraction from the PA-Sal coupled column was incubated with Vmw65 and labelled DNA probe in the presence of an equivalent volume of each of the salt eluted fractions. Lanes a and b, HeLa WCE incubated in the absence or presence of Vmw65, respectively; lanes c to g, excluded extract incubated with Vmw65 alone (lane c) or containing 5  $\mu$ l of the 0.1, 0.25, 0.6, or 1 M KCl fraction (lanes d to g, respectively); lanes h and i, 10  $\mu$ l of 1 M KCl fraction incubated alone or with Vmw65, respectively; lane J, Vmw65 incubated alone. Lanes k and l are the same as lanes c and g, respectively, except that Vmw65 was not incubated in the fractions.

complex was observed following addition of Vmw65 to the incubation (lane i). When the excluded fraction from PA-Sal affinity column was incubated with 1.0 M KCl eluent in the absence of Vmw65, no further complexes were detected on the gel (lane k and l).

These results demonstrated the following important properties of the cellular factor required for VIC formation: first, the cellular factor that bound directly to Vmw65 is required for VIC formation with Vmw65 and Oct-1; second, this factor did not form a complex with DNA; and third, this factor did not form a stable complex with Oct-1 under the conditions of affinity chromatography. This cellular factor was designated as VCAF-1 (stands for Vmw65 complex assembly factor).

### 3.3.3 VCAF-1 is heat sensitive

The fraction containing VCAF-1 activity was heated at 80°C and 100°C for 2 minutes in a water bath, then added to mobility shift reactions containing Vmw65 and VCAF-1 depleted HeLa cell extract. The results showed that the heat treatment at both temperatures destroyed the ability of VCAF-1 to reconstitute VIC with VCAF-1 depleted extract and virion Vmw65. The heat sensitive property of this factor implicated that VCAF-1 was probably proteinaceous in nature (Figure 3.3.4).



**Figure 3.3.4** The cellular factor interacting with Vmw65 is heat sensitive

The excluded fraction from the PA-Sal coupled column was incubated with Vmw65 and DNA probe in the absence (lane a) or presence of 5  $\mu$ l of the 1 M KCl fraction that was untreated (lane b) or that was heated for 2 min at 80°C (lane c) or 100°C (lane d).

### 3.3.4 SDS-PAGE analysis and silver stain

Whole cell extract, the excluded fraction from the PA-Sal conjugated affinity column and the eluent of different salt concentrations were analyzed by SDS-PAGE electrophoresis. The gels were silver stained to examine the specific bands that might correspond to the VCAF-1 activity. The results of silver staining failed to uncover any specific band from the VCAF-1 active fractions (Figure 3.3.5). Therefore, further purification was attempted in order to obtain a significant amount of VCAF-1 for characterization and cloning.

### 3.4 Purification of VCAF-1

Because the protein concentration in the 1 M KCl eluted fraction was too low to be detected by ordinary protein assays, such as Bio-Rad, and the silver stain the SDS-PAGE gel of this fraction failed to identify a specific band that corresponded to the VCAF-1 activity, conventional procedures of protein purification were necessary with a larger amount of starting material prior to the application on the affinity column. During the process of protein purification, the VCAF-1 activity was monitored by mobility shift assay using the VCAF-1 depleted HeLa cell extract.

The VCAF-1 activity was demonstrated to be mostly present in the nuclear extract, therefore, it was subsequently used in the purification of VCAF-1. For each preparation, 3 L



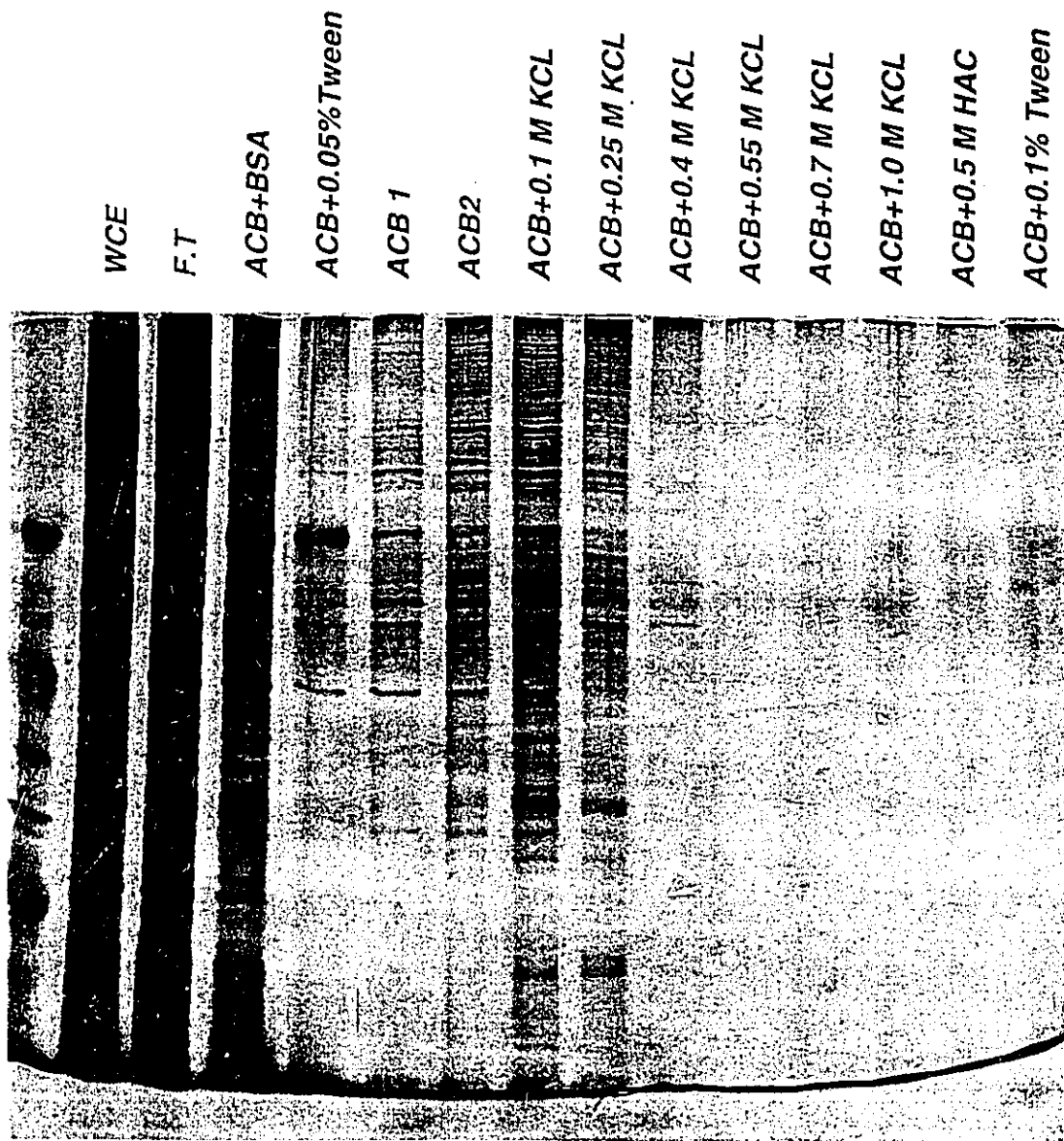


Figure 3.3.5 VCAF-1 was unable to be detected by SDS-PAGE and silver stain

Fractions of HeLa whole cell extract chromatographed on the column with CNBr-activated Sepharose coupled with PA-Sal were resolved on a 10% SDS-PAGE gel and silver stained.

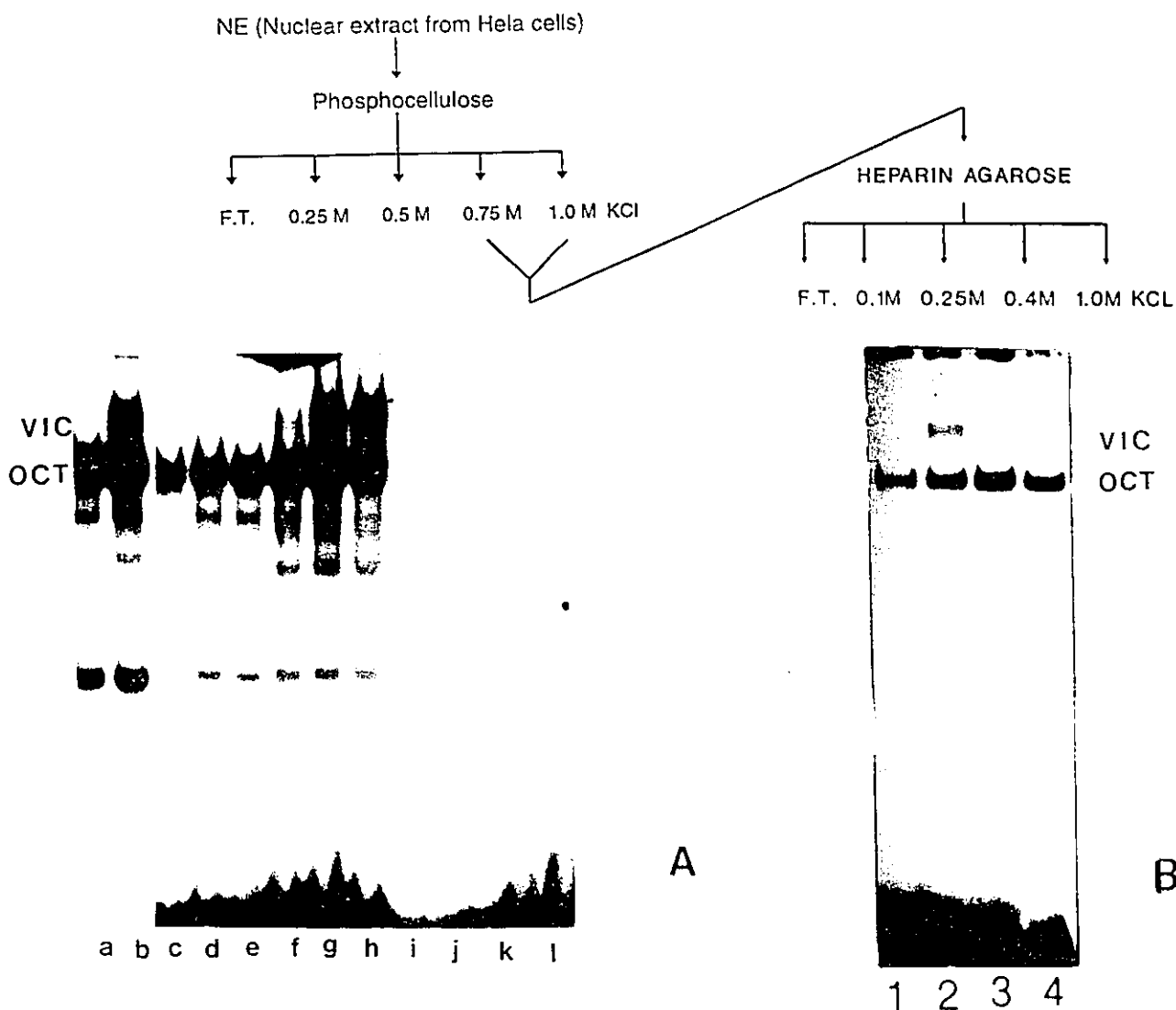
of HeLa cell suspension was grown and nuclear extract was made using a modified Dignam's procedure (1983). The S-100 fraction in preparation of nuclear extract contains little VCAF-1 activity, instead, it contains an additional activity that was able to enhance the formation of VIC. This VIC enhancing activity was later characterized by Werstuck and Capone (1993).

#### 3.4.1 Phosphocellulose chromatography

Phosphocellulose was utilized as the first step for purification because it absorbs a wide range of proteins and the bound material can be eluted by various conditions. 3 ml of pre-treated phosphocellulose was packed into a Bio-Rad column and equilibrated with buffer D (Dignam et al., 1983). To the column, 85 mg (6 ml) of HeLa nuclear extract was loaded and the flow through was collected and reapplied twice. The column was washed with buffer D until the O.D. at 280 nm was less than 0.20. The bound material was fractionated by each of 15 ml of buffer D containing 0.25 M, 0.5 M, 0.75 M and 1 M KCl. Bio-Rad assay was used to determine the protein concentration in each fraction (Table 3.4.1).

Mobility shift assays were carried out using VCAF-1-depleted HeLa cell extract and the fractions eluted from the phosphocellulose column. The VCAF-1 activity was found bound to the column at pH 7.9 and eluted by 0.75 to 1 M KCl concentration (Figure 3.4.1 A).





**Figure 3.4.1 Purification of VCAF-1**

**Panel A.** HeLa nuclear extract (NE) was fractionated by phosphocellulose (PC) column as shown. Lanes a and b contained 10  $\mu\text{g}$  of NE alone (a) or in the presence (b) of 0.05  $\mu\text{g}$  of Vmw65 to show the formation of VIC. The flow through (10  $\mu\text{g}$ ) from the PC column generated the Oct-1/DNA complex but not VIC when incubated with probe in the absence (lane c) or presence (lane e) of Vmw65. The flow through was incubated with Vmw65 and 5  $\mu\text{l}$  of salt fractions from 0.25, 0.5, 0.75 and 1 M KCl (lanes e to h, respectively). Lanes i to l that contained the same salt fractions as lanes e to h were incubated with Vmw65 in the absence of the flow through from PC column.

**Panel B.** PC fractions containing 0.75 and 1 M KCl eluent were pooled and fractionated on a heparin agarose column as indicated. Shown here, 5  $\mu\text{l}$  of fractions eluted from heparin agarose column by 0.1 to 1 M KCl salt (lanes 1 to 4, respectively) were incubated with excluded fraction from PC column and Vmw65.

### 3.4.2 Heparin agarose

The VCAF-1 active fractions from the PC column were combined and dialysed against buffer D and applied on a Heparin agarose affinity column (Bio-Rad), which was pre-equilibrated with buffer D. The column was washed with buffer D to an OD < 0.2 and the bound material was eluted with each of buffer D containing 0.25, 0.5, 0.75 and 1 M KCl. The VCAF-1 activity was present in 0.25 M KCl fraction (Figure 3.4.1 B).

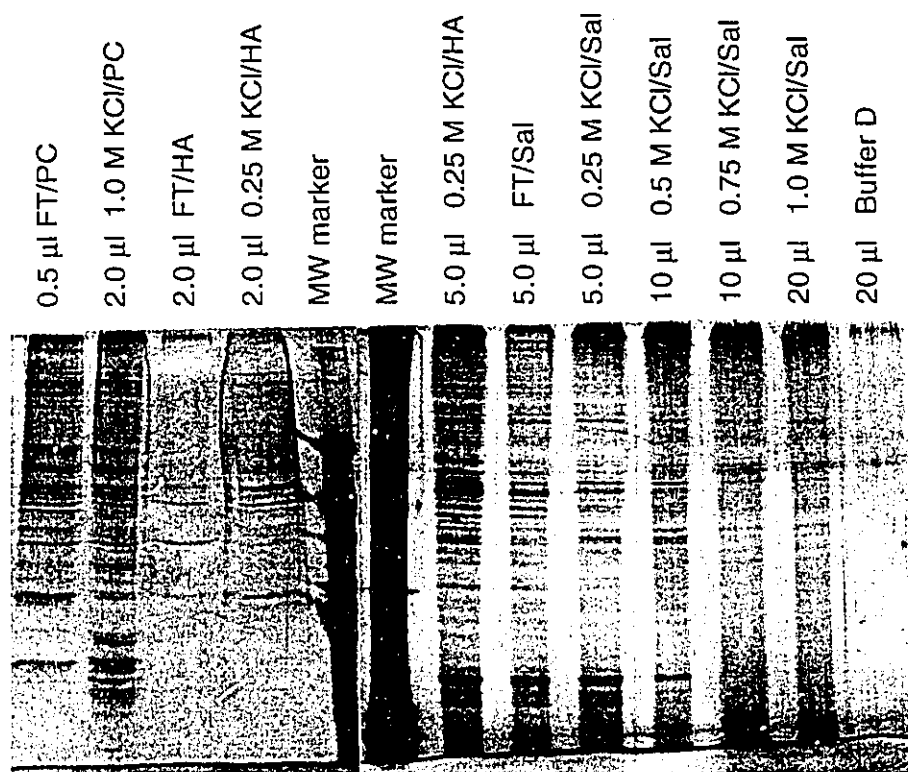
### 3.4.3 Affinity chromatography

The final step for the purification of VCAF-1 was carried out using Vmw65 affinity chromatography as described in previous sections of this chapter with some modifications. The affinity column of CNBr-activated Sepharose coupled with protein A fused Sal fragment of Vmw65 was pre-washed with: 1) 10 column volumes of buffer D; 2) buffer D containing 100  $\mu$ g/ml BSA; 3) buffer D containing 100  $\mu$ g/ml BSA and 0.05% NP40; 4) buffer D. The partially purified VCAF-1 from the heparin agarose column was dialysed against buffer D and applied on the affinity column at the flow rate of 0.2 ml/min. The application was repeated twice. The column was washed sequentially with 10 column volumes of buffer D, buffer D containing 0.05% NP40 and buffer D. The bound material was stepwise eluted with 5 column volumes of buffer D plus 0.25, 0.5, 0.75 and 1.0 M KCl. The VCAF-1 activity was present in the high salt eluent. However, when 20  $\mu$ l of the VCAF-1 active

fractions (5 times greater than required for VIC assembly) were resolved on SDS-PAGE gels and silver stained, we were still unable to identify the specific band for VCAF-1 (Figure 3.4.2). This implied that VCAF-1 was present in mammalian cells at very limited amount.

#### 3.4.4 Econo-Pak Q cartridge

Because of the low yield of VCAF-1 from phosphocellulose and heparin agarose purification procedure, other approaches were used for the initial steps of VCAF-1 purification. The Econo-Pak Q (Bio-Rad) cartridge filled with anion exchanger DEAE-cellulose was used as the first step for the purification of VCAF-1. 50 mg of Hela nuclear extract prepared as described above was applied on the Econo-Pak Q cartridge pre-equilibrated with buffer D. The column was washed with 5 bed volumes of buffer D and the bound material was eluted with 100 ml of buffer D containing linear KCl gradient of 0.1 to 1 M. The flow rate was 1 ml/min and each 1 ml fraction was collected. The VCAF-1 activity of each fraction was examined by mobility shift assay in the presence of Vmw65 and the POU domain of Oct-1 (the preparation of PA-POU will be discussed in the next chapter). VCAF-1 was present in the fractions between 250 and 350 mM KCl (Figure 3.4.3).



**Figure 3.4.2** Fractions subsequently fractionated on phosphocellulose, heparin agarose and PA-Sal affinity columns were resolved by an SDS-PAGE gel followed by silver staining. Content for each lane is indicated on the top of the gel.

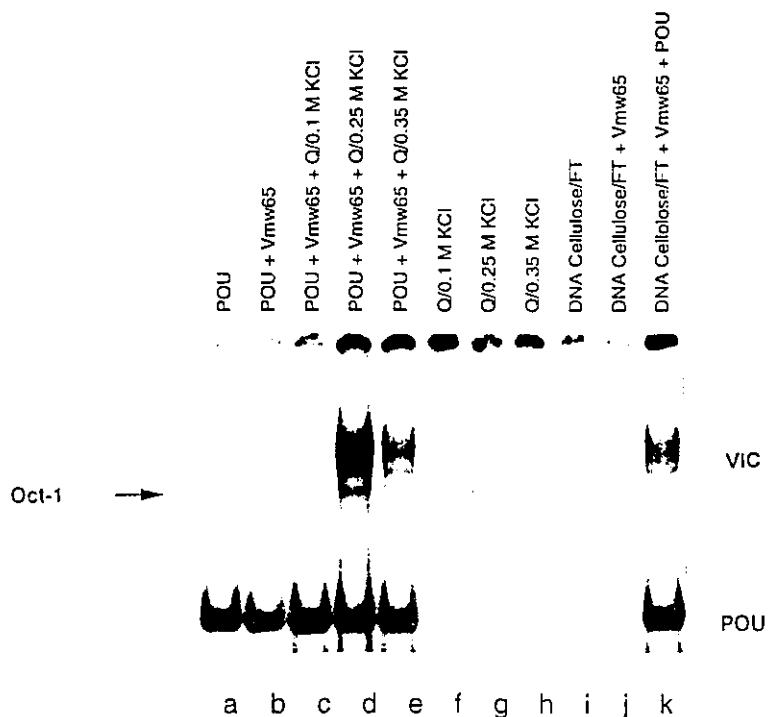
#### 3.4.5 DNA cellulose (VCAF-1 does not bind to DNA)

When VCAF-1 active fractions were examined using mobility shift assays, it was found that a weak Oct-1 activity co-purified with VCAF-1 in the Econo-Pak Q column fractions. To remove the Oct-1 activity, fractions containing VCAF-1 activity were pooled, dialysed against buffer D and applied on a native DNA-cellulose column (Pharmacia) pre-equilibrated with buffer D. The application was repeated three times. The flow through was collected and the bound material was eluted with buffer D containing 0.25 to 1 M KCl gradient. Mobility shift assay with these fractions found that the VCAF-1 activity was mostly present in the flow through while Oct-1 bound to the DNA cellulose column (Figure 3.4.3). This result further indicated that VCAF-1 does not possess DNA binding activity to either a specific or general DNA sequence.

#### 3.4.6 Affinity chromatography

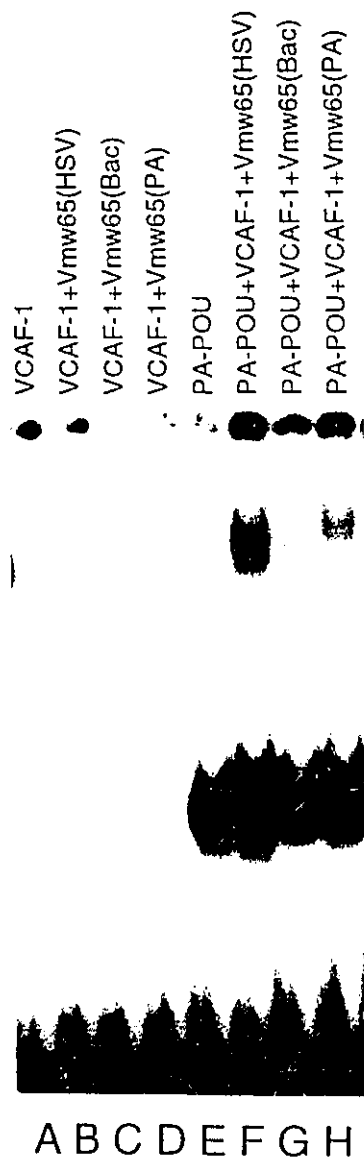
The VCAF-1 active fractions from DNA cellulose columns were combined, dialyzed with buffer D, then applied on PA-Sal coupled affinity column as described in section 3.4.3. The mobility shift assay by incubating the VCAF-1 containing fractions with *E. coli* expressed Oct-1 POU domain and Vmw65 from different sources demonstrated that VCAF-1 was able to generate VIC with Oct-1 and different sources of Vmw65 (Figure 3.4.4).





**Figure 3.4.3 VCAF-1 does not interact with DNA**

The fractions from Econo-Q column by KCl salt gradient were examined on mobility shift gel for VCAF-1 activity. Lane a: 0.1  $\mu$ g of PA-POU; lane b: PA-POU + 0.05  $\mu$ g of Vmw65 from baculovirus; lanes c, d, e: same as lane b except the addition of 4  $\mu$ l of eluted fractions corresponding the KCl concentration at 0.1, 0.25 and 0.35 M, respectively. Lanes f to h: same amount of KCl eluted fractions as lanes c to e incubated with the probe alone. After applied to native DNA-cellulose column, the flow through was incubated alone (lane i), and with Vmw65 (baculovirus) (lane j), as well as with Vmw65 and PA-POU (lanes k). The arrow indicates the remaining Oct-1.



**Figure 3.4.4** VCAF-1 formed VIC with Vmw65 and POU domain of Oct-1

Four  $\mu$ l of VCAF-1 purified from Vmw65 affinity column was incubated with the TAATGARAT DNA probe alone (lane A) or in the presence of Vmw65 from HSV extract, Baculovirus expression, or protein A fusion protein (lanes B to D, respectively). Lane E: PA-POU; lanes F to H were the same as lanes B to D with the addition of PA-POU.

### 3.5 Summary

Using an affinity column coupled to an *E. coli* expressed Vmw65 Sal fragment-protein A fusion, a cellular factor, other than Oct-1, was identified from HeLa cell extract. This factor binds to Vmw65 independently and is required for the assembly of Vmw65 dependent complex. It was, therefore, named VCAF-1 (for Vmw65 complex assembly factor). Further study in this thesis has revealed some properties of VCAF-1.

VCAF-1 possesses no direct DNA binding activity to either the TAATGARAT motif or general sequences. It does not interact with Oct-1 in the presence or absence of DNA. This study also found that, in the absence of DNA target, Oct-1 was unable to form a complex with Vmw65, directly or indirectly. Furthermore, VCAF-1 was present at very limited amounts in HeLa cells. Therefore, the role of VCAF-1 in the assembly of the Vmw65 induced complex was speculated to mediate the interaction between Vmw65 and DNA-bound Oct-1.

We have also investigated some biochemical properties of VCAF-1. VCAF-1 is proteinaceous in nature since it is completely inactivated by heat treatment and it binds to a heparin protein affinity column. It is negatively charged at normal physiological condition (pH 7.9) as determined by the fact that it interacts with DEAE-Sephacel and phosphocellulose but not with CM-Sepharose. This characteristic indicated that it might interact with positively charged regions of Vmw65.

I had experienced some difficulties in the purification of VCAF-1. The biggest problem I faced was the protein concentration in the VCAF-1 active fraction was so low that it could not be detected by the ordinary protein assay method such as Bio-Rad. Even the SDS-PAGE electrophoresis of the VCAF-1 containing fraction followed by silver staining resulted in many co-purified bands, none of which distinguishably corresponded to the VCAF-1 activity.

After we published our findings of VCAF-1, several other laboratories have also tried to purify and characterize this factor, notably, one at the Massachusetts Institute of Technology led by Dr. P. Sharp and one at the Cold Spring Harbor Laboratory led by Dr. W. Herr.

Kristie and Sharp (1993) reported that they had purified a cellular factor (C1) from HeLa nuclear extract. They started with 347 g of HeLa cell pellet and purified C1 9,000 fold by multi-step chromatography including 1) phosphocellulose; 2) Q Sepharose; 3) Mono Q; and 4) Mono S. A set of polypeptides ranging from 68 kDa to 135 kDa was obtained from this procedure. Therefore, they suggested that the C1 factor was a multiprotein complex of these polypeptides. They also pointed out the most significant functional characteristic of the C1 factor was its high affinity interaction with Vmw65 in the absence of the other components of the VIC. It was thus suggested that the formation of the C1- $\alpha$ TIF (VCAF-1-Vmw65) complex was the

initial step of assembling the Vmw65-dependent complex. The C1- $\alpha$ TIF (VCAF-1-Vmw65) complex then recognized the Oct-1/DNA element complex and formed the full VIC complex, which activated the transcription of IE genes. The protein-protein interaction between C1 and  $\alpha$ TIF might play regulatory roles in the activation of HSV IE genes. More recently, they reported that the C1 factor contains a family of polypeptides, which are all derived from a single large open reading frame (Kristie et al., 1995; also see below).

At Cold Spring Harbor, Wilson et al. (1993) purified HCF (host cell factor) from HeLa cell extract to near homogeneity using WGA (wheat germ agglutinin) agarose affinity chromatography followed by Mono Q or Mono S column. They found that HCF was composed of six related polypeptides with molecular masses from 110 to 150 kDa and a minor 300 kDa polypeptide. They sequenced some polypeptides and used the amino acid sequences to make degenerate probes, with which they isolated the cDNA encoding HCF from human cDNA libraries. The complete cDNA of HCF has been cloned and sequenced (Figure 3.5.1). It contains a large open reading frame (ORF) having 2035 amino acid codons, within which eight repeats of a 26 amino acid motif have been found. The 300 kDa polypeptide and the six related smaller polypeptides are all encoded by this single large ORF. The presence of the 300 kDa polypeptide and the lack of significant splicing products indicated that the processing occurred post-translationally. More recently,

Wilson et al. (1995) reported that the repeat of HCF acts as a signal for proteolytic cleavage, by which the central portion of the nascent polypeptide is removed resulting in mature HCF polypeptides containing amino- and carboxyl terminal fragments of the 300 kDa precursor protein.

The identification of cellular factor VCAF-1 was a very important step towards our understanding of the mechanism involved in transcriptional control of eukaryotic genes through multi-protein complexes. It is now more clear that the activation of selective genes in eukaryotic cells is orchestrated by distinct combinations and arrangements of different transcriptional factors. The identification of VCAF-1 has provided us a premise to study how these proteins are appropriately selected and assembled onto the *cis*-acting target sites to facilitate activation of transcription. The findings that we first reported about VCAF-1 and its properties have been confirmed by later studies in other laboratories (Katan et al., 1990; Kristie and Sharp, 1990, 1993; Stern and Herr, 1990; Wilson et al., 1993). Our approach was one of the first examples of using affinity chromatography to isolate transcription factors by protein-protein interaction and is a strategy that is now widely used.

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MASAVSPANLPAVLLQPRNKRVCWSPVPRPRHGRHRAVAIKELIVVFGGNEGIVDELHVYNTATNQWFIPAVRGOIPPGCAAYGFVCDGTRLLVFGGH 100
VEYGYKYNOLYELQASRWENKRLKAKTPKNGPPPCRLGHSFSLVGNKCYLFGGLANSEDPKNNIPRYLNDLYLELRPGSGVVAWDIPITYGLPPRR 200
ESHTAVVYTEKDNKSKLVIYCGHSGRCLGDLWTLIDITLWNKPSLSGVAPLPRSLHSATTIGNKMYVFGGVPLVMDDVKVATHEKWKCTNTLACLH 300
LDTHAWEITLMDTLEDNIPRARAGHCAVAINTRLYIWSGRDGYRKAWNQVCCKDLWYLETEKPPPPARVQLVRANTNSLEVSNGAVATADSYLLQLQKY 400
DIPATAATASPTPNPVSPANPPKSPAPAAAAVQPLTQVGTILLQAAAPAPPTTTTIQVLPVPGSSISVPTAARTQGVPAVLKVTGPQATTGTPL 500
VTNRPASQAGKAPVTVTSIPAGVRMVPTQSAQGTIVGSSPQMSQHAALAAAAATQKIPPSAPTVLSVPAGTTIVKIMAVTPGTTTLPATVAVASSPY 600
MVSNPATRMLKTAQAQVGTSSATNTSTRPIITVHKSQGTVAQQAQVTTVVGGVTKITLVKSPISVPGGSALEISNLGKVMVVQTKPVQTSAVTQQ 700
ASTGPVTIIQTKGPLPAGTILKLVTSADCKPTTIIITTTQASGAGTKPTILGISSVSPSTTXPGTTTTIKTIHMSAIIQAGATGVTSPPGKSFITIT 800
TKVMTSGTGAPAKIITAVPKIATGHGQQGVTVVVKGAPGQPGTILRTVPHGGVRLVTPVTVSAVKPAVTTLVKGTGVTTLGTVTGTVSTLAGAGGH 900
STASASLATPIITLGTIATLSSQVINPTAITVSAQTTLTAAGGLTPTITMOPVSOPTQVTLITAPSGVEAQVHDLVYSILASPTTEQPTATVTIADSG 1000
QGQVQPGTVTLVCSNPPCETHETGTTTATTIVANLGGHPQPTQVQVFCDRQEAASLVTSTVGGQNGSVVRVCSNPPCETHETGTTTATTATSNMAG 1100
QHCCSNPPCETHETGTTTATTAMSSVGANHQDARRACAAAGTAVIRISVATGALAAAQGSKSCQTRQTSATSTTMTVMAATGAPCSAGPLLGPSMARE 1200
PGGRSPAFLVQLAPLSSKVRLLSSPSIKDLPAGRHSVAVSTAAMTRSSVGCAGEPRMAPVCESLQGGSPSTTVTAL EALLCPSATVTQVCSNPPCETHETG 1300
TTNTATTSNAGSAQRVCSNPPCETHETGTTTATTATSHGGTQPEGGQPPAGRPCE THQTTSTGTTMSVSGALLPDATSSHRTVESGLEVAAPSVT 1400
PQAGTALLAPFPTQRVCSNPPCETHETGTTTATTATSNHSSNQDPPAASOQGEVES TQGDVNISSSAITTTVSTL TRAVTTVTQSTPVGPSVPP 1500
PERLQVSPGPRQLPPRQLLQSASTALMGESA EVL SASQTPELPAAVDLSSTCEPSSQESAGS AVVATVVVQPPPTQSEVDQLSLPQELMAEAQAGTT 1600
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KSPDGAHLTWEPPSVTSKGIIEYSVYLAIQSSOAGGELKSSTPAQLAFNRVYCGPSPSCLVQSSSLSHAHIDYTKPAIIFRIARHEKGYGPATQVRWL 2000
QETSKDSSGTPANKRPMSSPEMKSA PKKSKADGQ 2035

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pep-1  
 pep-2  
 pep-3  
 pep-4  
 pep-5  
 pep-6  
 pep-7  
 pep-8  
 pep-9  
 pep-10  
 pep-11  
 pep-12  
 pep-13  
 repeat-1  
 repeat-2  
 repeat-3  
 repeat-4  
 repeat-5  
 repeat-6  
 repeat-7  
 repeat-8  
 α rHCF  
 pep-14  
 pep-15  
 pep-16  
 pep-17  
 pep-18  
 pep-19  
 α rHCF ←

Figure 3.5.1 The complete Amino acid sequence of HCF (VCAF-1) predicted from the human HCF-1 cDNA

HCF is encoded by a single open reading frame that encodes 2035 amino acids. The primary sequence is shown in single letter code. Each HCF repeat is indicated by a line above the sequence (two degenerate repeats are indicated by broken lines). Adapted from Wilson et al., 1993.

## CHAPTER 4

### ANALYSIS OF VMW65-INDUCED COMPLEX (VIC) ASSEMBLY WITH PURIFIED COMPONENTS

#### 4.1 Background

As described in the previous chapter, to activate the transcription of HSV-1 IE genes, Vmw65 must mediate the formation of a specific DNA-binding complex with two cellular factors, Oct-1 and VCAF-1. A detailed understanding of the properties of Vmw65 associated with the stepwise assembly of VIC and the distinct interactions among these factors could provide important insights into the cooperative assembly of transcriptional activation complexes, and help to elucidate the mechanism of selective transcriptional activation of eukaryotic genes. *E. coli* expressed fusion proteins could facilitate the investigation of distinct protein-protein and protein-DNA interactions involved in the assembly of VIC: 1) The purified proteins simplify the interactions so that the assembly of VIC can be studied in a stepwise manner and *in vitro*. 2) The cloning and sequencing of the protein coding region of Vmw65 (Dalrymple et al., 1985) has made it possible



to investigate functionally important domains of Vmw65 by the manipulation of its cDNA. The requirement for VIC assembly had been located within the amino-terminal 400 amino acid residues (Greaves and O'Hare, 1989; Werstuck and Capone, 1989b). 3) Two other components required for VIC formation have been identified as cellular factors, Oct-1 and VCAF-1. These factors have been affinity purified, and furthermore, Oct-1 has been cloned (Herr et al., 1988; Sturm and Herr, 1988).

Previous studies by Werstuck and Capone (1989b) using *E. coli* expressed Vmw65 and mutants thereof demonstrated that protein A fused Vmw65 was capable of forming VIC with HeLa cell extract. However, HeLa cell extract contains all the host cell factors and cannot be used to study the exclusive interactions among each individual factor. As mentioned in the previous chapter, the excluded fraction of HeLa cell extract from Vmw65-Sal affinity column was able to form the Oct-1-DNA complex at the TAATGARAT probe without the formation of VIC in the presence of Vmw65. Although this VCAF-1 depleted fraction of HeLa cell extract contains Oct-1 and can be used to identify the presence of VCAF-1, or monitor the purification of VCAF-1 by the addition of VCAF-1-containing fractions, the HeLa cell excluded fraction from the Vmw65 affinity column also contains all cellular proteins except those bound to Vmw65 directly. Thus, we could not rule out the possibility that some other cellular factors that did not bind to the

affinity column were also required for VIC assembly. In other words, incomplete results could be obtained if the VCAF-1 depleted fraction was used to study each individual requirement for VIC assembly or to monitor the purification of VCAF-1. Thus, homogenous Oct-1 was essential for our investigation of the stepwise assembly of VIC with viral and cellular components.

Therefore, the first objective in studying the functional cooperative assembly of VIC was to establish an assay system by which the specific interactions involved in this process could be detected using purified components.

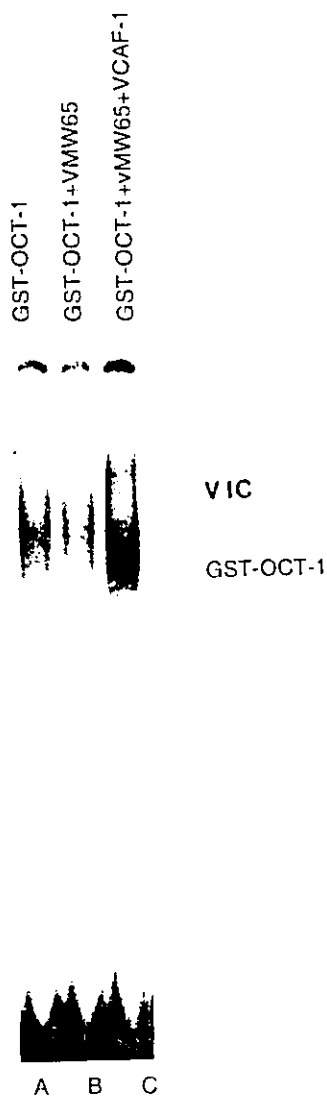
#### 4.2 Generation of Oct-1 and Vmw65 fusion proteins

As a first step to studying protein-protein and protein-DNA interaction, the two cloned components of Vmw65 induced complex, Oct-1 and Vmw65, were expressed as fusion proteins with different *E. coli* expression systems. When choosing a system, the yield, stability and biochemical function (proper folding) of products; cloning strategies (cloning sites and orientations); as well as the application of fusion proteins have to be considered. Therefore, we tested different systems to find out the best condition for expression of the genes to be studied.

#### 4.2.1 Expression of Oct-1 as a GST fusion protein

Oct-1 encodes a polypeptide of 743 amino acids, within which a 160 amino acid bipartite POU domain DNA-binding structure has been identified (Sturm et al., 1988). To purify Oct-1, the entire coding sequence of the Oct-1 gene was subcloned from plasmid pBSOct-1<sup>+</sup> (provided by Dr. W. Herr, Cold Spring Harbor) into an *E. coli* expression vector (pGEX) so that it could be expressed as a fusion protein with GST (glutathione S transferase). The construct was transformed into *E. coli* strain DH-5 $\alpha$ , and the expression of GST-Oct-1 was induced with IPTG. The fusion protein was subsequently purified using a glutathione-Sepharose affinity column (Pharmacia).

Mobility shift assays were carried out with this *E. coli* expressed GST-Oct-1 fusion protein. Like Oct-1 from nuclear extract, the GST-Oct-1 has independent DNA-binding activity to the TAATGARAT probe. However, when virion Vmw65 was present in the incubation, no additional shift that corresponded to VIC was observed, as expected (Figure 4.2.1). Consequently, when VCAF-1 was added to the reaction, it generated the VIC shift. One problem with using the GST-Oct-1 fusion protein is that it has a very large molecular weight. The intermediate products in VIC assembly, if there are any, would be difficult to observe. As a result, the cooperative interaction between Vmw65 and Oct-1 cannot be well resolved by



**Figure 4.2.1** Recombinant Oct-1 is able to form VIC with Vmw65 and VCAF-1

0.5  $\mu\text{g}$  of GST-Oct-1 was incubated with DNA probe alone (lane A), or in the presence of 0.05  $\mu\text{g}$  of Vmw65 from HSV extract (lane B) or both Vmw65 and VCAF-1 (lane C).

gel shift assay with this product. In addition, the presence of degradation byproducts during the purification complicates the experimental results. We therefore constructed a POU domain derivative of Oct-1 since it was shown that the POU domain was both necessary and sufficient for Oct-1 to selectively bind to the TAATGARAT DNA element and mediate the formation of a multi-component complex with Vmw65 (Sturm and Herr, 1988; Kristie and Sharp, 1990; Herr and Cleary, 1995).

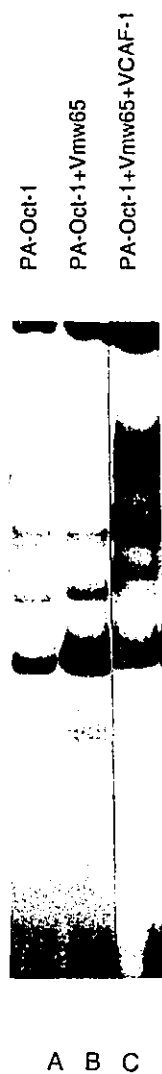
#### **4.2.2 The POU domain fusion protein is sufficient for DNA-binding and VIC assembly**

The POU domain is the structural motif shared by the DNA-binding proteins of the POU domain family. It is characterized by two independent DNA-binding structures, namely, POU-specific and POU-homeo subdomains, linked by a loop structure (Herr et al., 1988). This particular structure confers both selectivity and flexibility in DNA-binding and protein-protein interactions to POU domain proteins.

The DNA fragment that encodes the POU domain (amino acids 271-441) and an additional 151 amino acids downstream was cloned into the *Bam*HI site of pRIT2T. This construct, referred to as pRIT-Oct-1, was expressed as a protein A fusion protein and purified as described in Chapter 2. However, the mobility shift assay using this recombinant protein showed that it was a very unstable product and contained a number of

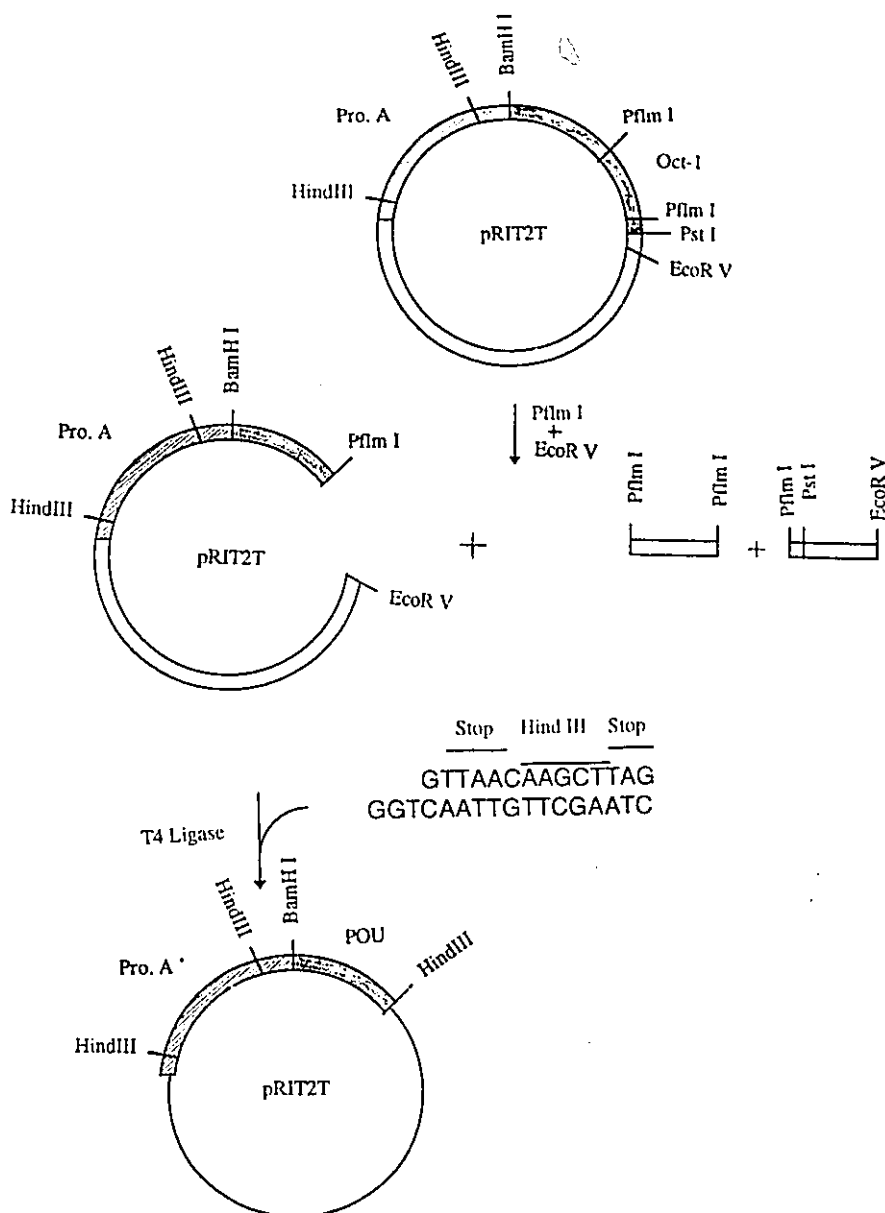
co-purified degradation byproducts, which generated multiple bands on the gel (Figure 4.2.2). As a result, we subcloned just the POU domain.

pRIT-Oct-1 was digested with *PflmI* located just downstream from the second helix of the POU-homeo domain and *EcoRV* to remove the downstream sequence (amino acids 592-441) of Oct-1. The fragment containing the plasmid backbone and the POU domain was then ligated together with an oligonucleotide linker that contains two stop codons in different frames and a *HindIII* site (Figure 4.2.3). As a result, the fragment containing amino acids 271-441 of Oct-1, which corresponds to the POU domain, was cloned downstream from the protein A gene. The plasmid was transformed into an *E. coli* N4830-1 strain. The POU domain of Oct-1 was expressed as a protein A fusion protein (PA-POU) and purified with an IgG Sepharose affinity column as described in Chapter 2 (Figure 4.2.4 A). Mobility shift analysis with PA-POU demonstrated that it binds to the TAATGARAT DNA probe and generates a single major DNA-binding complex on the gel. When incubated with Vmw65 and VCAF-1, the PA-POU is capable of forming VIC, in addition to the POU-DNA-binding complex (Figure 4.2.4 B). Because the latter experiments showed that the PA-POU was unstable after long storage, the POU domain was also subcloned into the pGEX vector and purified as a GST fusion protein (GST-POU).



**Figure 4.2.2 PA-Oct-1 generates multiple complexes**

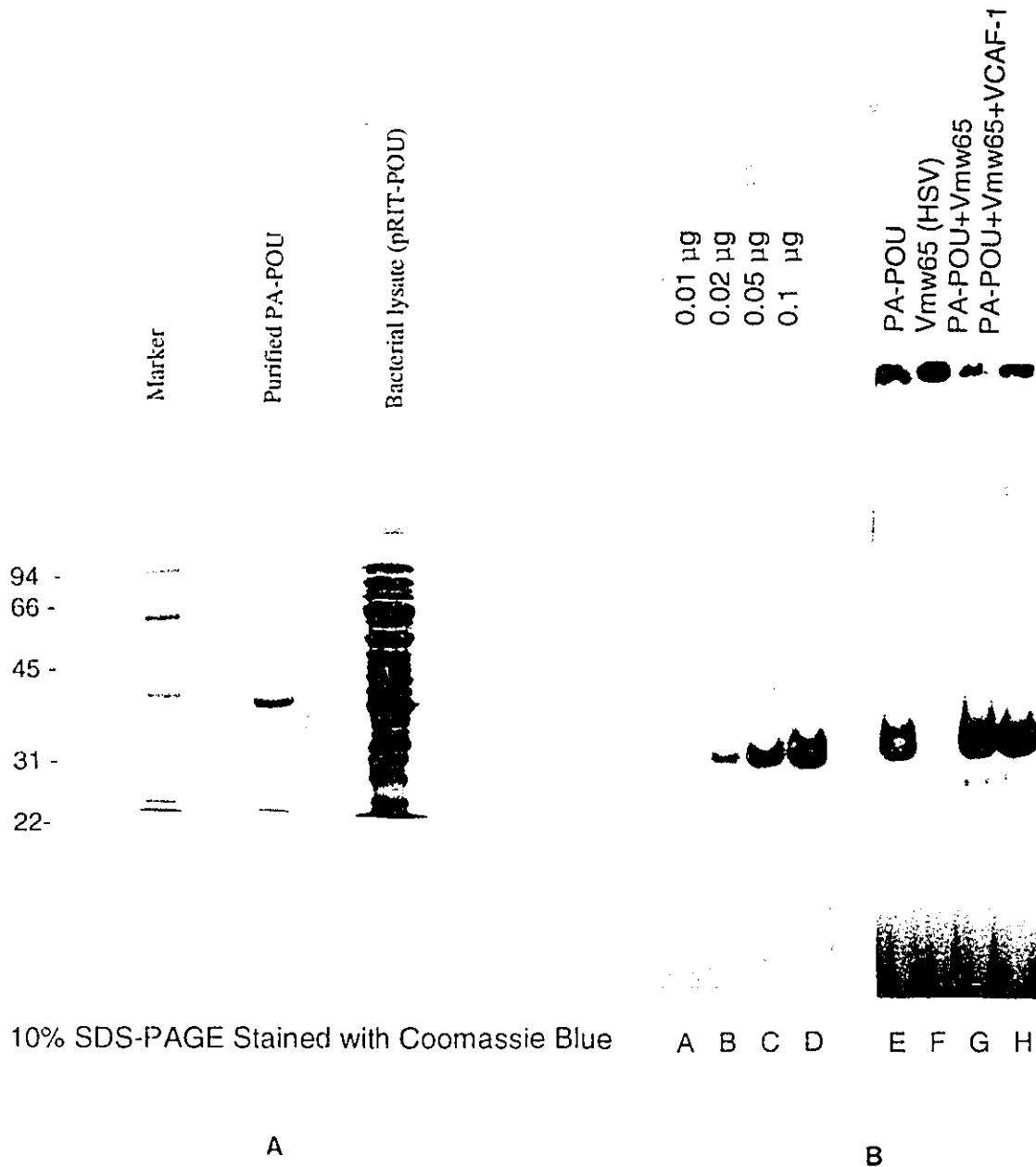
The fragment containing the POU domain of Oct-1 and downstream sequences was expressed as a protein A fusion (PA-Oct-1) and used in mobility shift assays: lane A, 0.5  $\mu$ g of PA-Oct-1 was incubated with labelled DNA probe; lane B, the same amount of PA-Oct-1 incubated in the presence of 0.05  $\mu$ g of Vmw65 from HSV; lane C, the same as lane B with the addition of VCAF-1.



**Figure 4.2.3 Subcloning of the POU domain**

The construct pRIT-Oct-1 was double digested with *Pflm* I and *EcoRV* to remove the downstream sequence from the POU domain. The fragment containing the plasmid backbone and the POU domain of Oct-1 was ligated to a linker that contains stop codons in two frames and a *HindIII* site. The construct was transformed into the *E. coli* N4830 strain.





**Figure 4.2.4 PA-POU forms VIC with Vmw65 and VCAF-1**

**Panel A:** PA-POU was induced by temperature shifting (lane 3) and purified with an IgG-Sepharose affinity column (lane 2); lane 1 shows molecular weight marker.

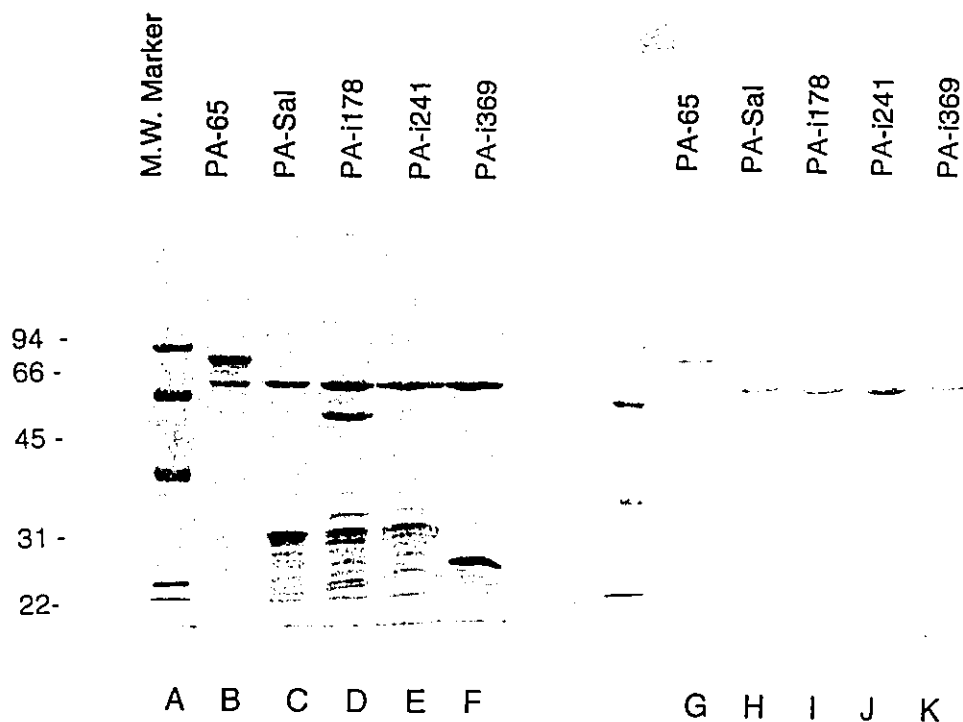
**Panel B:** mobility shift assays showed that the purified PA-POU generates a single major DNA-binding complex which increases with the amount of PA-POU. Lanes A to D contained the DNA probe and 0.01, 0.02, 0.05 and 0.1 µg of PA-POU, respectively. PA-POU was able to form VIC with Vmw65 and VCAF-1. The DNA probe was incubated with 0.1 µg of PA-POU alone (lane E); 0.05 µg of Vmw65 from HSV extract alone (lane F); PA-POU and Vmw65 (lane G); PA-POU, Vmw65 and VCAF-1 (lane H), respectively.

### 4.3 Vmw65 fusion proteins

We have successfully identified the cellular factor VCAF-1 and subcloned the POU domain of Oct-1. In order to investigate the stepwise assembly of the Vmw65-induced complex with purified components, Vmw65 was expressed as fusion proteins of protein A, glutathione-S-transferase (GST) and maltose binding protein (MBP). These purified proteins were tested for their capabilities to form VIC with HeLa cell extract or with affinity-purified VCAF-1 and PA-POU.

#### 4.3.1 Vmw65 protein A fusion proteins

Vmw65 and linker-insertion derivatives previously shown to affect VIC formation were subcloned into the multiple cloning site of the *E. coli* expression vector pRIT2T (Werstuck and Capone, 1989b). These constructs were expressed and purified as protein A fusion proteins as described in Chapter 2. The purified proteins were run on an SDS-PAGE gel to examine their sizes and purity. However, the SDS-PAGE gel showed that there were always some extra polypeptides co-purifying with protein A and the fusion proteins (Figure 4.3.1 A). Econo Q cartridge (Bio-Rad) chromatography was used to purify protein A fusion proteins to greater homogeneity (Figure 4.3.1 B). The functional analysis of these fusion proteins will be discussed in section 4 of this chapter.



**Figure 4.3.1** Expression and purification of Vmw65 and linker insertion mutants as protein A fusion proteins

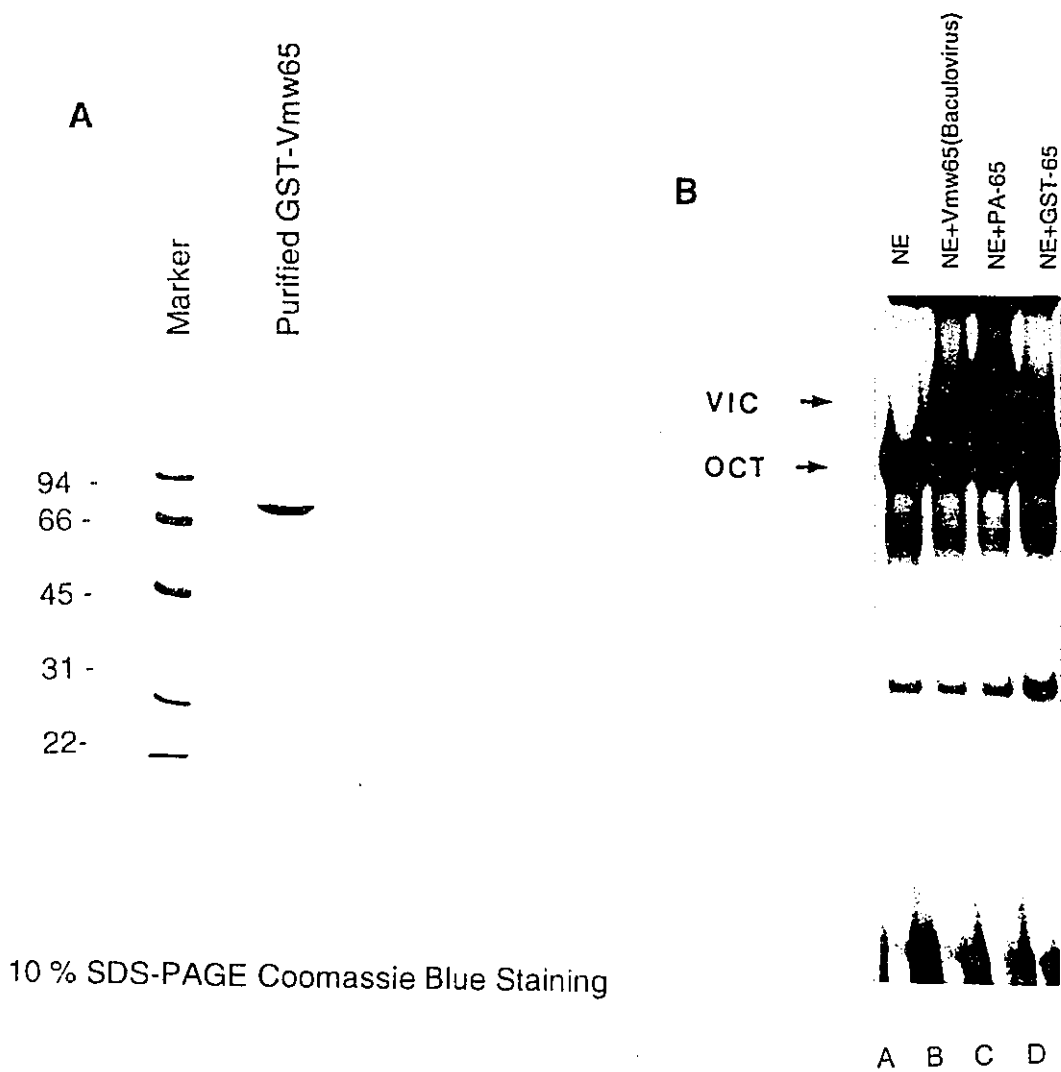
**Panel A:** Vmw65 and mutants were purified by IgG Sepharose chromatography and resolved on an SDS-PAGE gel followed by Coomassie Blue staining, lane A: M.W. marker; lanes B to F: PA-65, PA-Sal, PA-i178, PA-i241, and PA-i369 respectively.

**Panel B:** The IgG-purified PA-Vmw65 and mutants were further purified with Econo Q cartridge (Bio-Rad). Shown here, the fusion proteins purified with the Econo Q column were resolved on an SDS-PAGE gel and stained with Coomassie Blue. Lanes G to K are PA-65, PA-Sal, PA-i178, PA-i241, and PA-i369 respectively.

#### 4.3.2 Vmw65 GST fusion proteins

Because the natural folding status of fusion proteins could be altered by the presence of carrier protein, other fusion protein systems should be used to confirm the observations obtained by using one particular fusion protein. Besides protein A, GST and MBP fusion protein systems were also chosen to express Vmw65 and mutants. The advantage of GST and MBP fusion protein systems is that the precursor proteins can be removed by proteolytic cleavage with thrombin or Factor Xa.

The induction and purification of GST fusion proteins were carried out as described in Materials and Methods (Chapter 2). Before large-scale preparation, a pilot experiment was performed to determine the best conditions for protein expression. The production of fusion proteins was monitored by running the bacterial lysate on an SDS-PAGE gel at different times post-induction. The purified GST fusion proteins were examined on an SDS-PAGE gel. It was found that the GST fusion proteins were usually more stable and had less degradation than their protein A counterparts, probably due to the gentle induction and elution conditions (Figure 4.3.2 A). Like protein A fusion proteins, the GST-fused Vmw65 was able to form the Vmw65-dependent complex on a mobility shift gel when incubated with HeLa nuclear extract (Figure 4.3.2 B).



**Figure 4.3.2 Vmw65 expressed as a GST fusion protein is capable of forming VIC**

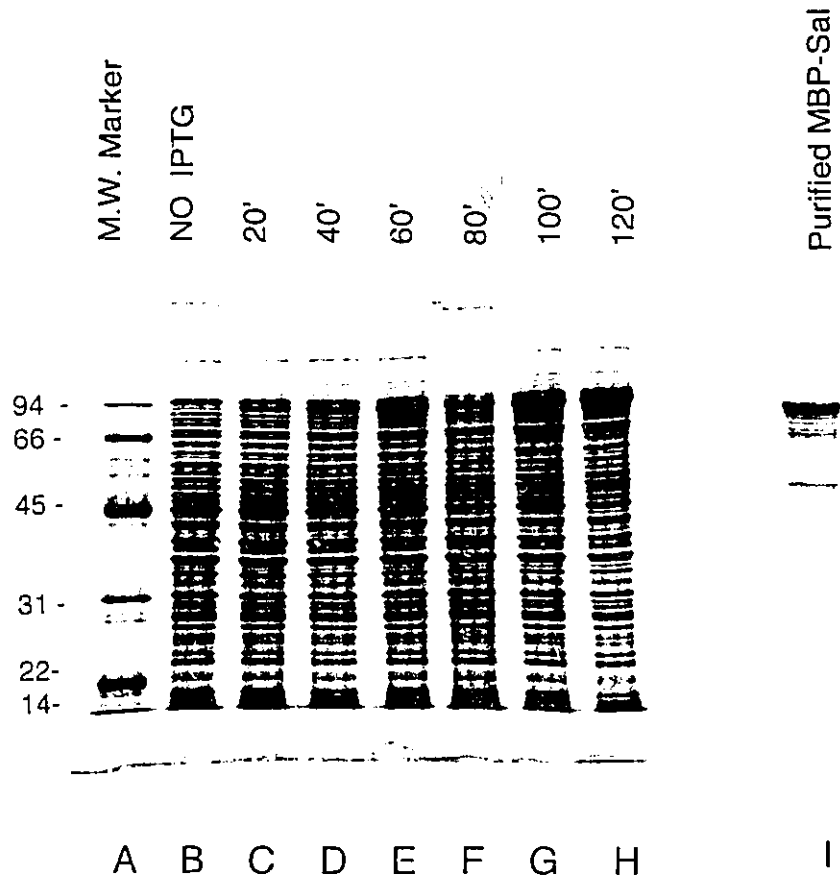
**Panel A:** pGEX-65 was induced by adding IPTG and purified glutathione Sepharose affinity column. Purified GST-Vmw65 was run on an SDS-PAGE and silver stained.

**Panel B:** Vmw65 derived from baculovirus (lane B), or expressed as protein A (lane C), or GST (lane D) fusion proteins were incubated with the DNA probe in the presence of HeLa nuclear extract. Lane A contained HeLa NE alone.

#### 4.3.3 Vmw65 MBP fusion proteins

The genes of Vmw65 and mutants were also cloned into the multiple cloning site of the *E. coli* expression vector pMAL-c2. To determine the best conditions of inducing fusion proteins by IPTG, a pilot experiment was carried out by collecting bacteria at 20, 40, 60, 80, 100 and 120 minutes after induction. The cell lysate was analyzed by SDS-PAGE. At 100 minutes, the expression of fusion protein reached its peak (Figure 4.3.3A). The MBP fusion proteins were purified from bacterial lysate with amylose affinity resin as described in chapter 2 (Figure 4.3.3B).

MBP fusion proteins have strong affinity to amylose resin. Our experiment showed that the fusion protein is still able to interact with the beads in a buffer containing 1 M salt concentration. As a result of this interaction, the fusion protein bound amylose resin can be directly used as an affinity ligand to purify VCAF-1 or study protein-protein interactions involved in Vmw65-dependent complex assembly. Because the affinity ligands (MBP) are naturally bound to the matrix, the fused proteins are mostly exposed to the surface of the beads, rather than randomly coupled as on CNBr-activated Sepharose. The MBP fusion proteins purified by affinity chromatography usually have higher efficiency and specificity than those of Vmw65-coupled to CNBr-activated Sepharose. The stability of the MBP fusion protein is



**Figure 4.3.3 The induction of MBP fusion protein by IPTG**

MBP-Sal was induced by the addition of IPTG to a final concentration of 0.3 mM. Bacterial cells were collected every 20 minutes post-induction. Each lysate was run on an SDS-PAGE gel and stained with Coomassie Blue. Lane A: M.W. marker; lane B: bacterial lysate before adding IPTG; lanes C to H are bacterial lysate prepared at 20, 40, 60, 80, 100 and 120 minutes after the addition of IPTG. Lane I shows the MBP-Sal purified with amylose resin.

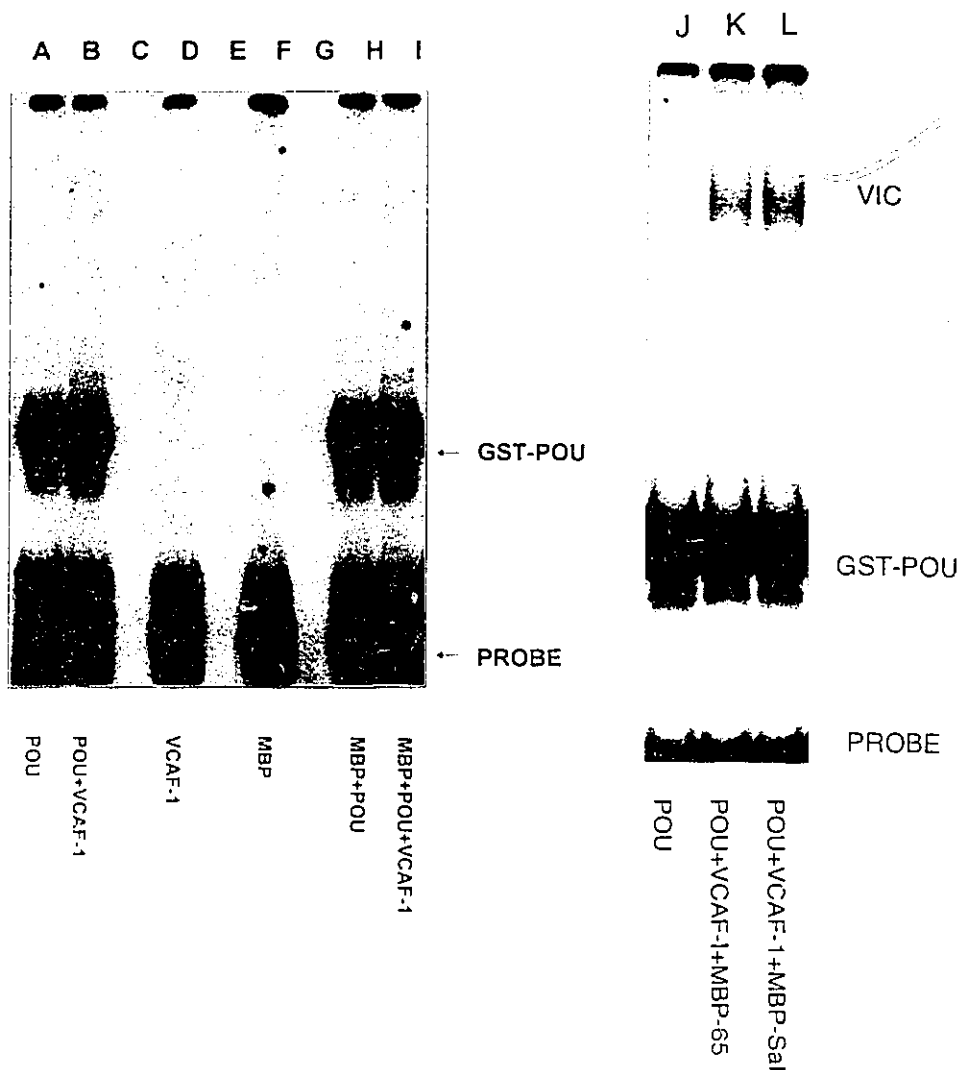
comparable to that of GST fusion proteins. Like protein A and GST fusion proteins, MBP fused Vmw65 is capable of generating Vmw65-dependent complex on a mobility shift gel when it is incubated with nuclear extract, or purified POU domain and VCAF-1 (Figure 4.3.4).

Vmw65 expressed as fusion proteins by all three fusion protein systems were functionally active in mediating the formation of VIC with HeLa cell extract or with POU and VCAF-1. However, each system has its own advantages and disadvantages. For instance, pRIT is a highly efficient expression system, but the product is less stable due to the high inducing temperature and the acidic elution condition. Degradation by products are usually co-purified by the column. The fusion proteins expressed by pGEX and pMAL are relatively stable but the yields vary depending on the inserted gene. A special advantage for the pMAL system is that the binding between fusion protein and ligand is very strong even at high salt concentration. This has provided an approach for making an affinity column by directly coupling the fusion protein to the resin.

#### **4.4 Investigation of protein-DNA and protein-protein interactions involved in VIC assembly using protein A fusion proteins and purified VCAF-1**

As previously shown (Werstuck and Capone, 1989b), the protein A-fused Vmw65 displays the same properties as the





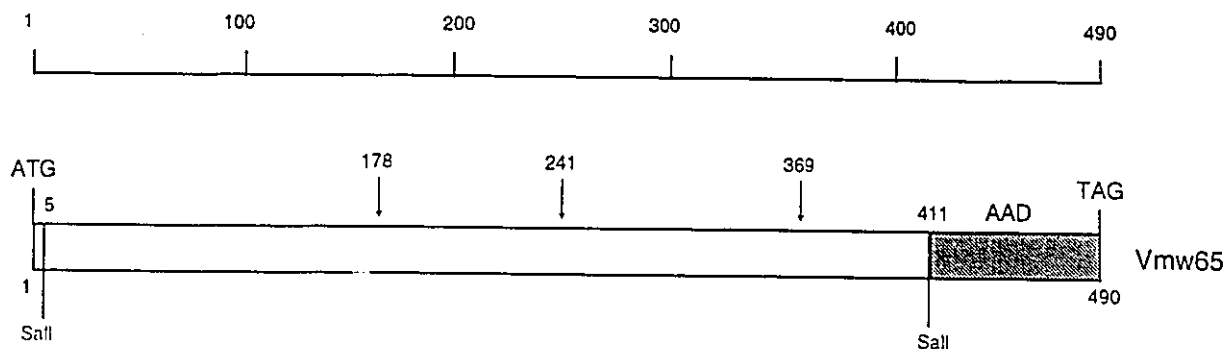
**Figure 4.3.4** MBP-65 and MBP-Sal are capable of forming VIC with POU and VCAF-1

Mobility shift analysis demonstrated that the carrier protein (MBP) had no effect on the binding activity of POU and VCAF-1, neither did it generate VIC in the presence of POU and VCAF-1 (lanes A to I: containing 0.5  $\mu\text{g}$  of MBP and/or 0.05  $\mu\text{g}$  of GST-POU and/or VCAF-1 as indicated). However MBP fusion proteins to Vmw65 and its Sal fragment were able to generate VIC with DNA probe in the presence of GST-POU and VCAF-1 (lanes J to L: containing 0.05  $\mu\text{g}$  of GST-POU alone or 0.5  $\mu\text{g}$  of MBP fusion proteins and VCAF-1 as indicated).

Vmw65 purified from virus particles in terms of generating the Vmw65-induced complex. By examining VIC formation *in vitro* and activation *in vivo* with various linker insertion mutational derivatives (Werstuck and Capone, 1989a,b), they found that linker insertion mutants i178, i241 and i369 (at positions 178, 241, and 369 within the amino-terminal domain, respectively, Figure 4.4) had exhibited some interesting characteristics: 1) When incubated with HeLa cell extract *in vitro*, i241 formed the complex as efficiently as wild type Vmw65, while i178 formed the complex very poorly and i369 did not form any detectable complex. 2) The *in vitro* VIC formation abilities of these mutants seemed consistent with their activities to activate IE gene expression *in vivo*. 3) Mutant i241 had approximately 2.5-fold higher inducing efficiency than that of wild-type Vmw65 (Werstuck and Capone, 1989a,b). However, the VIC formation abilities of these mutants had never been tested with purified components.

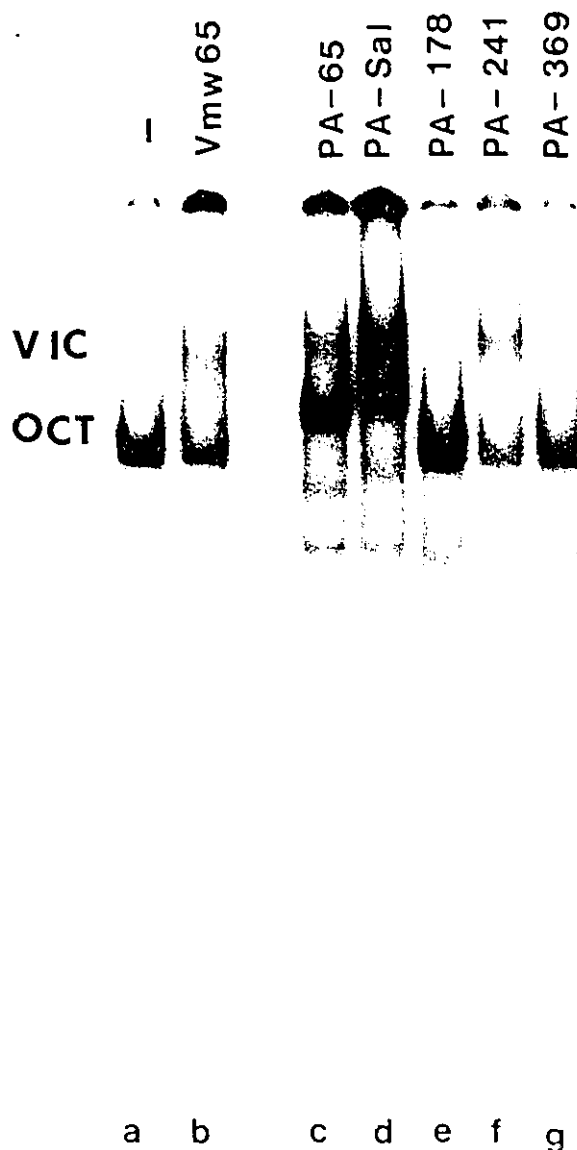
Therefore, these three insertions were utilized to further investigate various requirements for protein-protein and protein-DNA interactions involved in the assembly of multi-component complexes with purified VCAF-1 and PA-POU.

As demonstrated in Figure 4.4.1, mutants PA-i178 and PA-i369 abolished the ability of Vmw65 to mediate the formation of VIC when incubated with HeLa cell extract (lanes e and g). In contrast, PA-65, PA-Sal, or PA-i241 were able to



**Figure 4.4** Schematic representation of Vmw65

The coding region of Vmw65 is represented with the start and stop codons indicated. Amino acid numbers are shown at the top of the Figure. The carboxyl-terminal acidic activation domain is represented by the solid box and the amino-terminal domain, which is responsible for VIC formation, is represented by the open box. BamHI linkers were inserted at various sites (indicated by arrows) within the N-terminal domain (Werstuck and Capone, 1989b). The SalI sites from which the Sal fragment was generated are indicated.



**Figure 4.4.1** Mobility shift analysis of VIC assembly with PA-65 and mutant fusion proteins

HeLa nuclear extract (8  $\mu$ g) was incubated with DNA probe in the absence (lane a) or presence of 0.1  $\mu$ g of Vmw65 derived from baculovirus or 0.1  $\mu$ g of various protein A-Vmw65 fusion proteins, as indicated at the top of the figure (lanes b to g).

form the Vmw65 induced complex (lanes c, d and f, respectively). Interestingly, both PA-65 and PA-Sal also altered the shift of Oct-1 to a slower migration but the PA-i241 did not result in this alteration. This result indicated that a selective cooperative interaction might exist between Vmw65 and Oct-1.

We wished that the stepwise assembly of VIC *in vitro* using purified components could define the requirements of these interactions in the assembly of functional active complexes.

#### 4.4.1 Vmw65 has intrinsic DNA-binding activity

Vmw65 had been considered to have no DNA-binding activity to either a specific sequence or to DNA in general (Marsden *et al.*, 1987; McKnight *et al.*, 1987; Preston *et al.*, 1988). However, Kristie and Sharp (1990) reported that Vmw65 purified from baculovirus or expressed as a protein A fusion protein displayed a weak DNA-binding activity to the TAATGARAT element at high concentration of protein. This DNA-binding activity is sequence-specific and requires the presence of the GARAT sequence. An explanation for this phenomenon is that the naturally folded Vmw65 shields the DNA-binding site of Vmw65 and the interactions of cellular factors or the *E. coli* expressed fusion proteins alter the conformation of the molecule so that it can interact with the DNA element.

We tested DNA-binding activity by incubating protein A-fused Vmw65 and its mutants (Figure 4.3.1) with  $^{32}\text{P}$ -labelled TAATGARAT probe. When the amount of fusion protein in the reaction was increased to an amount about 20 times greater than that used to generate VIC, a specific DNA-binding product was obtained on a mobility shift gel (Figure 4.4.2). PA-65 and PA-Sal were able to efficiently bind to DNA (lanes a and b, respectively). Furthermore, PA-Sal, which lacks the AAD, had higher affinity to DNA than the full-length protein. One possibility is that the acidic domain of Vmw65, which is not required for DNA-binding, may interfere with the protein-DNA interaction due to the negative residues that repel the negatively charged DNA backbone. PA-i369 bound to DNA with less efficiency (lane e) and PA-i178 was unable to bind to DNA (lane c). Interestingly, PA-i241 was unable to bind to DNA although it was capable of forming VIC with HeLa cell extract (see Figure 4.4.1).

It is worth mentioning that the weak intrinsic DNA-binding activity of Vmw65 varies with the source of protein. Vmw65 expressed as protein A and MBP fusion proteins efficiently generated DNA-binding complexes (MBP fusion proteins will be discussed in detail in the following chapters), while Vmw65 derived from baculovirus or from HSV, or expressed from the pGEX vector, were much less efficient to bind to DNA (Figure 4.4.3). It is possible that the carrier

PA-65  
PA-Sal  
PA-178  
PA-241  
PA-369

132

a b c d e

**Figure 4.4.2 Protein A-Vmw65 fusion proteins have intrinsic DNA-binding activity**

Two  $\mu\text{g}$  of PA-Vmw65 and mutant fusion proteins, as indicated at the top of the figure, were incubated with the TAATGARAT probe and the protein-DNA complexes were resolved on a 3.5% native gel. The intrinsic DNA-binding activity was observed with PA-65, PA-Sal and PA-i369 (lanes a, b and e), while PA-i178 and PA-i241 did not form a protein-DNA complex (lanes c and d).

2 µg PA-Sal  
5 µg PA-Sal  
10 µg PA-Sal  
2 µg GST-Sal  
5 µg GST-Sal  
10 µg GST-Sal



A B C D E F

**Figure 4.4.3** The intrinsic DNA-binding activity of Vmw65 varies by source

Equivalent amounts of PA-Sal or GST-Sal fusion proteins were incubated with the TAATGARAT probe. Shown here, lanes A to C contained 1, 2.5, 5, and 10 µg of PA-Sal; lanes D to F contained the same amounts, as A to D, of GST-Sal.



proteins influence the natural folding of Vmw65 causing the DNA contacting domain of Vmw65, which is normally buried, to be exposed on the surface. Previous studies had also demonstrated that DNA-binding activity of baculovirus-produced Vmw65 could be increased by denaturing then renaturing the protein (Kristie and Sharp, 1990). Therefore, protein-protein interactions with cellular factors may stabilize the Vmw65-DNA complex and play a role in regulating complex assembly.

#### 4.4.2 Investigation of cooperativity of Vmw65 with DNA-bound Oct-1

The POU domain of Oct-1, which binds to the octamer sequence independently, is considered the driving force in directing the assembly of the multi-component complex (Stern et al., 1989, Stern and Herr, 1991). However, Oct-1 or its POU domain fail to interact with Vmw65 in the absence of DNA target (Kristie and Sharp, 1990; Xiao and Capone, 1990). Although Vmw65 does not have efficient DNA-binding activity, it is able to recognize and bind to the POU domain of Oct-1 in the presence of TAATGARAT DNA element *in vitro* (Stern and Herr, 1991). One explanation is that the Oct-1-Vmw65 interaction is DNA-binding site dependent. In the absence of DNA, the protein-protein contacts formed between the Oct-1 POU domain and Vmw65 are not sufficiently strong to maintain the stable interaction with Oct-1. The cooperative interaction of Vmw65 with DNA bound Oct-1 would serve to stabilize their

association (Stern and Herr, 1991). To test such a hypothesis, and to further localize the subdomain of Vmw65 that cooperatively interacts with the DNA-bound POU domain of Oct-1, mobility shift assays were carried out using *E. coli* expressed protein A fused POU domain (PA-POU) and protein A fused to Vmw65 and its mutants.

As demonstrated in Figure 4.4.4, when incubated with the TAATGARAT DNA probe at a level of 0.1  $\mu$ g (the amount of fusion protein required to form VIC with HeLa nuclear extract), PA-65, PA-Sal and PA-i369 were able to bind to DNA (lanes a, b and e, respectively) although the intensity was much less than that in Figure 4.4.2, in which 2  $\mu$ g of fusion proteins were used to show the intrinsic DNA-binding. The addition of PA-POU to the incubations resulted in cooperative DNA-binding activity of PA-65 and PA-Sal (lanes g and h). However, such cooperative DNA-binding between Vmw65 and Oct-1 was not observed with the insertions PA-i178, PA-i241 and PA-i369. The case of PA-i369 drew special attention because it has intrinsic DNA-binding activity but failed to cooperatively interact with PA-POU. This result implied that the DNA-binding domain of Vmw65 and the domain involved in cooperative interaction with POU domain of Oct-1 might be located in different regions of Vmw65, and the insertion at 369 disrupted the structure required for the interaction with Oct-1. The

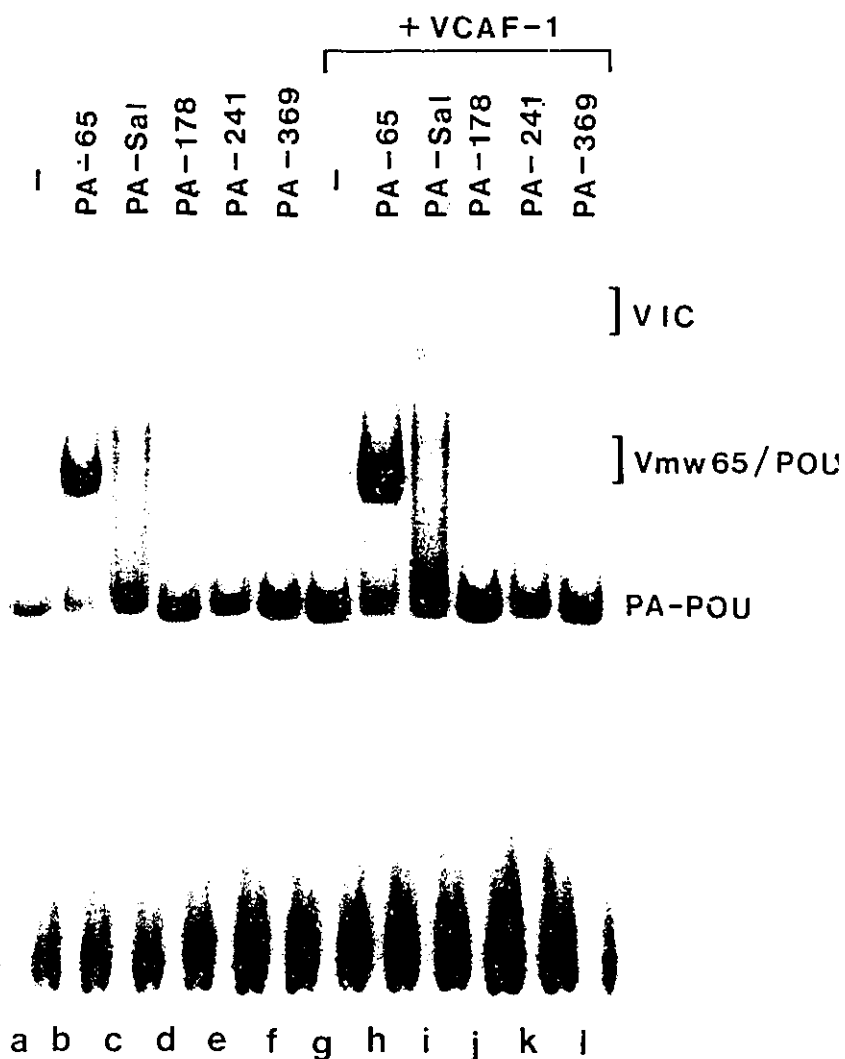


insertion mutant 241 that had neither intrinsic DNA-binding nor cooperative Oct-1 binding activity, was still able to generate VIC with HeLa nuclear extract or in the presence of purified PA-POU and VCAF-1. This indicated that the binary interaction between Vmw65 and DNA-bound Oct-1 might be disrupted, but had no effect on cooperative protein-protein interactions in the multi-component DNA-binding complex when all components co-existed.

#### 4.5 The role of VCAF-1 in Vmw65-induced complex assembly

Although VCAF-1 binds to Vmw65 directly without the presence of Oct-1 and DNA element, there is no evidence that it possesses either independent DNA-binding or Oct-1 binding activity (Xiao and Capone, 1990; Kristie and Sharp, 1993; Wilson et al., 1993). In order to understand the role of VCAF-1 in the assembly of the multi-component DNA-binding complex, experiments designed to assemble VIC in a stepwise manner using *E. coli* expressed POU domain of Oct-1, Vmw65 (and its mutants) and affinity-purified VCAF-1 with the TAATGARAT DNA probe were carried out.

When added into a reaction containing only PA-POU and DNA probe, VCAF-1 had no effect on the DNA-POU complex since neither the mobility of this complex was altered nor was an additional shift observed (Figure 4.4.5, lane g). However, when VCAF-1 was added to the reaction containing PA-POU, Vmw65, and the TAATGARAT DNA probe, a specific complex, in



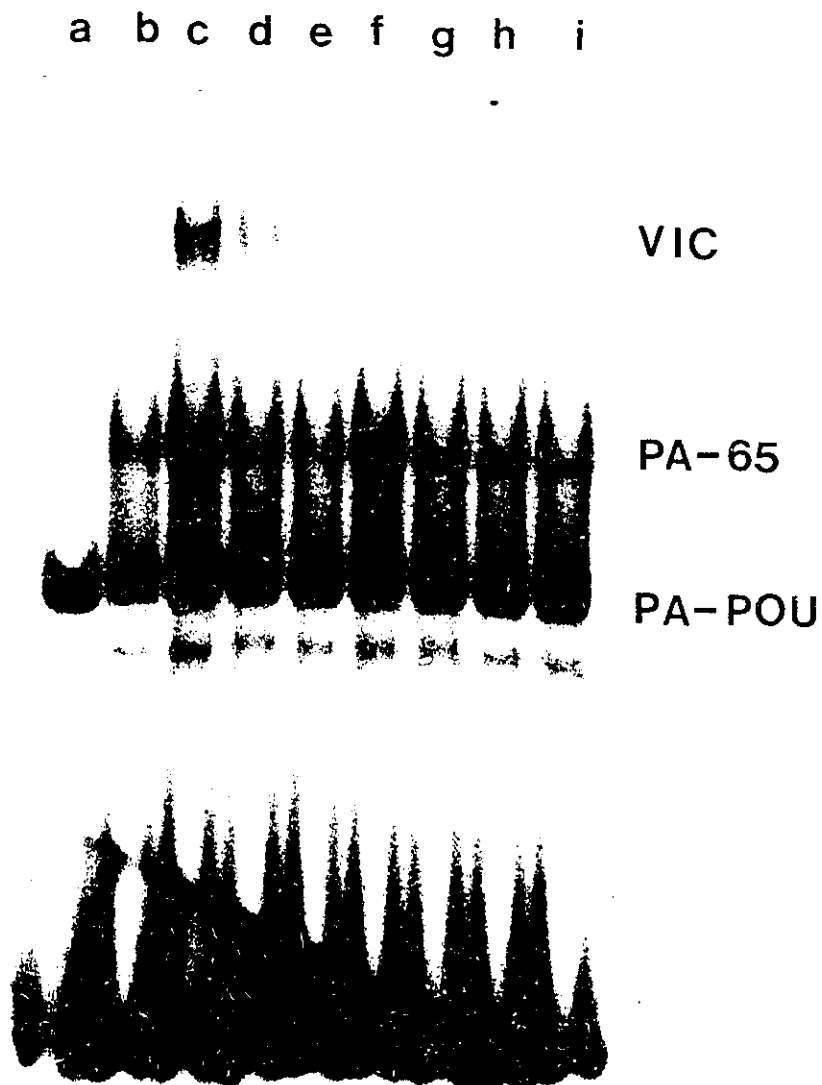
**Figure 4.4.5** PA-65, PA-Sal and PA-i241 were able to generate VIC in the presence of PA-POU and VCAF-1

PA-POU (0.1  $\mu$ g) was incubated in the absence (lanes a and g) or presence of 0.1  $\mu$ g of the various protein A fusion proteins as indicated at the top of the figure. Lanes a to f are identical to lanes g to l, except the latter contained 4  $\mu$ l of VCAF-1.

addition to POU and POU-Vmw65, was generated (lane h). It was further observed that PA-65 and PA-Sal as well as PA-i241 were able to mediate VIC formation with PA-POU and VCAF-1 (lanes h, i and k respectively), but PA-i178 and PA-i369 were unable to form VIC (lanes j and l). This result was consistent with previous observations with HeLa nuclear extract (Figure 4.4.1).

The fact that PA-i241, which did not bind to DNA nor interact with DNA bound POU-homeo domain, but formed VIC with VCAF-1 and PA-POU, demonstrated that the requirement for Vmw65 to independently bind to DNA or cooperatively interact with DNA-bound Oct-1 is not necessary for VIC assembly.

To further examine whether the independent interaction between VCAF-1 and Vmw65 is necessary for VIC assembly, a micro affinity assay was carried out using the IgG-Sepharose beads coupled to protein A fusion proteins of Vmw65 and its mutants. VCAF-1 was incubated with these affinity resins. The non-bound material was collected and tested for VCAF-1 activity by mobility shift assay with Vmw65 and PA-POU. The results (Figure 4.4.6) showed that PA-i178 and PA-i369 had no direct VCAF-1 binding activities (lanes g and i, respectively). These results, in combination with the results that PA-i178 and PA-i369 could not mediate VIC formation in the presence of VCAF-1 and PA-POU, suggest that VCAF-1 binding is necessary for VIC assembly indicating that VCAF-1 may mediate the interaction of Vmw65 with DNA-bound Oct-1 through



**Figure 4.4.6 Micro-affinity assay**

VCAF-1 was incubated with IgG-Sepharose affinity matrix containing immobilized protein A or PA-Vmw65 fusion proteins. The unbound material was collected and incubated with 0.05  $\mu\text{g}$  of PA-POU and 0.05  $\mu\text{g}$  of PA-65 to examine the presence of VCAF-1. Lane a, PA-POU alone; lane b, PA-POU and PA-65; lane c, same as lane b but containing 4  $\mu\text{l}$  of VCAF-1. Lanes d to i: PA-POU and PA-65 were incubated with 8  $\mu\text{l}$  of non-bound material from affinity matrix containing protein A (lane d), PA-65 (lane e), PA-Sal (lane f), PA-i178 (lane g), PA-i241 (lane h) and PA-i369 (lane i).

protein-protein interaction rather than promote direct Vmw65-DNA interaction. At least in one of the VIC assembly pathways, the pre-formed Vmw65-VCAF-1 complex is directed to the TAATGARAT *cis*-acting element by the DNA bound Oct-1 through the cooperative protein-protein interaction between Vmw65 and Oct-1.

#### 4.5 Summary

*In vitro* mobility shift assays with purified protein elements and a radiolabelled DNA target has provided a tool to study protein-DNA and protein-protein interactions in complex assembly. In this chapter, I describe the establishment of *E. coli* expression systems to express Vmw65 and mutant constructs as fusion proteins of protein A, GST and MBP. These fusion proteins have proved functionally active, and more importantly, effective for the study of distinctive properties of Vmw65 involved in the assembly of a specific DNA-binding complex. Like Vmw65 purified from a virally infected cell extract, all these Vmw65 fusion proteins are able to generate VIC with HeLa nuclear extract or with purified Oct-1 and VCAF-1.

The POU domain of Oct-1 is both necessary and sufficient to interact with the octamer element, and to mediate the assembly of VIC (Sturm and Herr, 1988; Scott *et al.*, 1989; Kristie and Sharp, 1990). Mobility shift gel analysis has demonstrated that *E. coli* expressed POU domain



fusion proteins function equivalently to the full-length Oct-1 in both binding to the octamer DNA element and the assembly of VIC. Because of the smaller size, the POU proteins have special advantages in the experiments, in which the uncoupling of protein-protein interactions in VIC assembly is needed.

We used several linker insertion mutants of Vmw65, previously demonstrated to have different effects on VIC formation with HeLa cell extract (Werstuck and Capone, 1989b), to examine various requirements for protein-protein and protein-DNA interactions involved in VIC assembly, including their intrinsic DNA-binding activity, cooperativity with DNA-bound Oct-1 and direct interaction with VCAF-1.

The finding that Vmw65 has sequence-specific DNA-binding activity when high protein concentrations are used implied that the sequence-specific interaction between DNA and Vmw65 might play an important role in directing the formation of transcriptionally active complex. The removal of the GARAT element from IE gene promoters not only disrupts binding by Vmw65 but also abolishes the activation of IE genes (Goding and O'Hare, 1988; Kristie et al, 1989; Werstuck, 1993). The weak DNA-binding activity of Vmw65 may be required to confer a proper arrangement for the complex to activate the downstream genes, but is not sufficient for either cooperative interaction with Oct-1 or VIC assembly, since PA-i369 was able to specifically bind to DNA but was defective in binding to VCAF-1 and cooperating with Oct-1 for complex formation.

Studies thus far had not been able to detect a direct interaction between Vmw65 and Oct-1 in the absence of DNA (Kristie and Sharp, 1990; Xiao and Capone, 1990). This work showed that Vmw65 was able to cooperatively interact with the POU domain of Oct-1 in the presence of DNA target. However, a linker insertion at amino acid 369 had weak intrinsic DNA-binding activity but failed to cooperatively interact with DNA-bound Oct-1. This indicates that there are different requirements for DNA-binding and cooperative Oct-1 interaction. The region around 369 is sensitive to the interaction with Oct-1 but may not be required for DNA-binding.

The role of VCAF-1 in VIC assembly was also investigated. The mutants that independently interacted with VCAF-1 were also generated VIC complex *in vitro*. With the mutants tested, PA-i241 did not have detectable intrinsic DNA-binding activity or cooperativity with Oct-1, but it was capable of forming VIC with both VCAF-1 and Oct-1. One possibility is that PA-i241 was able to bind to DNA independently, but the protein-DNA complex is very unstable, hence, the cooperative interactions with both Oct-1 and VCAF-1 are needed to induce an appropriate conformation in PA-i241 so that it can interact with DNA when assembled into the complex. Because the interaction of Vmw65 with VCAF-1 is in accordance with VIC assembly, it is likely that the pre-formed Vmw65 and VCAF-1 complex is directed to the TAATGARAT site by the DNA-

bound Oct-1 to form a functional transactivation complex.

The functional relevance of intrinsic DNA-binding activity of Vmw65 or cooperative interaction with DNA-bound Oct-1 is still unclear, since there is no evidence that Vmw65 itself or the Vmw65-Oct-1 complex is transcriptionally active *in vivo*. The amount of Vmw65 required for direct DNA-binding is significantly higher (10- to 100-fold) than that required for generating the Vmw65-induced complex with HeLa cell extract or with purified Oct-1 and VCAF-1. Furthermore, this study and a study by others (Kristie and Sharp, 1990) has found that the source of Vmw65 directly affects the efficiency of DNA-binding activity of Vmw65. The high concentration of Vmw65 is probably not physiologically significant since the transactivation of viral IE genes is negatively affected by the large amount of Vmw65 *in vivo* (Greaves and O'Hare, 1989). The high concentration of Vmw65 probably squelches away the cellular factor VCAF-1, which is present at very limited amounts in the host cell (this thesis).

More recent studies found that the expression of VCAF-1 varies at different stages of cell development indicating that VCAF-1 may play a regulatory role in cell proliferation as well as in the control IE gene expression (Wilson et al., 1995). Therefore, under physiological conditions, the interactions with Oct-1 and VCAF-1 may influence the DNA-binding activity of Vmw65 to ensure a greater degree of specificity and selectivity in transcriptional control of IE

genes. Compared to the abundant presence of Vmw65 in virion and the high level expression of Oct-1, the amount of VCAF-1 is very low, hence, the prior assembly of the VCAF-1-Vmw65 complex may play a regulatory role in subsequent activation of HSV-1 immediate early genes. Further investigation of the combinatorial role of Oct-1 and VCAF-1 in modulating structural and functional changes to Vmw65 would be particularly important for understanding the mechanisms involved in complex assembly and transcriptional activation (will be discussed in detail in the following chapters).

## CHAPTER 5

### MUTATIONAL ANALYSIS OF VMW65 -- STRUCTURE-FUNCTIONAL RELATIONSHIP AND THE PROPERTIES INVOLVED IN VMW65 INDUCED COMPLEX ASSEMBLY

#### Background

Mutational studies have revealed that the amino-terminal 400 amino acids of Vmw65 are necessary and sufficient for mediating Vmw65 induced complex assembly (Triezenberg et al., 1988; Greaves and O'Hare, 1989; Werstuck and Capone, 1989b). Using deletion of large segments and linker insertion Werstuck and Capone (1989a,b) had interrupted VIC formation and activation function of Vmw65. A synthetic peptide competition experiment had shown that peptide containing amino acids 360-373 was capable of disrupting VIC formation but whether this region of Vmw65 interacts with cellular factors could not be determined (Haigh et al., 1990). Stern and Herr (1991) mapped the Oct-1 binding domain of Vmw65 to amino acids 378-389 just upstream of the activation domain. They had also shown that a synthetic peptide containing amino acids 170-202 of Vmw65 had non-specific DNA binding activity (Stern and

Herr, 1991). Another mutational study of Vmw65 had shown that the amino terminal boundary for complex assembly was located between amino acids 49 and 75, while the carboxyl terminal boundary was located between residues 380-388 (Greaves and O'Hare, 1990).

These studies indicated some subregions within the amino terminal 400 residues involved in the formation of VIC. However, each individual interaction, including the intrinsic DNA binding, interacting with DNA bound Oct-1 and the interaction with VCAF-1, and its role in facilitating the assembly of transcriptionally active complexes had not been addressed. Although some discrete properties associated with Vmw65 in VIC assembly have been described in the previous chapter with Vmw65 fusion proteins and purified Oct-1 and VCAF-1, only a limited number of linker insertions were used. A more systematic mutational study was needed to investigate the requirement of individual protein-protein and protein-DNA interactions in the formation of complexes on responsive targets and their relevance to IE gene activation. This chapter is divided into 2 parts: part 1 will describe carboxyl terminal truncation mutagenesis; part 2 will be site-directed mutagenesis.

**Part 1. Mutagenesis Study with Carboxyl Terminal  
Truncations**

**5.1 Preparation of mutant fusion proteins**

**5.1.1 Construction of C-terminal truncation mutants**

Carboxyl terminal truncated mutants of Vmw65 were chosen as the first set of mutations, because the stepwise removal of amino acids from the carboxyl terminal could specifically target the potentially sensitive regions of Vmw65 responsible for each protein-protein and protein-DNA interactions.

pSPUTK65 containing the full length Vmw65 gene was used as starting material for the deletion mutagenesis. To create other truncation mutants, the pRIT-Sal (containing amino acids 4-411, constructed by G. Werstuck) was used. It was digested with PstI, which is located just downstream from SalI site, and another restriction enzyme at a specific site to remove further fragments downstream. The fragment containing the plasmid backbone and the amino terminal portion of Vmw65 gene was separated, blunt ended with T4 DNA polymerase, and ligated with T4 DNA ligase. The chosen truncation sites were: ApaI (amino acid 73), Eco47III (151), KpnI (181), NruI (200) and NaeI (290). As a result, a series of carboxyl terminal truncations (referred to as pRIT-T73, pRIT-T151, pRIT-T181, pRIT-T200 and pRIT-T290) were constructed and expressed as protein A fusion proteins.

Besides the above carboxyl terminal truncations,

previously prepared carboxyl terminal truncated mutants containing premature termination codons (constructed by Rob Wheatly) were also subcloned into the expression vector pRIT2T. pSPUTK plasmid harbouring carboxyl terminal truncations (pSPUTK-T250, pSPUTK-T299, pSPUTK-T335, pSPUTK-T369, pSPUTK-T379, and pSPUTK-T404) were digested with BglII, separated, and cloned into the BamHI site of pRIT2T.

Table 5.1 shows the carboxyl terminal truncation mutants obtained from the above two methods together with some previously made deletion mutations.

#### **5.1.2 Expression of Vmw65 and C-terminal truncation mutants as fusion proteins of maltose binding protein (MBP)**

Since the expression of protein A fused Vmw65 truncation mutants resulted in poor yields and severe degradation, the *E. coli* expression vector pMAL-c (New England Bio-Labs) was used to express Vmw65 and its truncation mutants as MBP fusion proteins. Vmw65 and its carboxyl terminal truncation mutants (T-250, T-299, T-335, T-369, T-379 and T-404) were subcloned into the pMAL-c vector. The full length Vmw65, T-299, T-379, and T-404 were separated from their original vector pSPUTK by cutting with BglII and cloned into the BamHI site of pMAL-c. In addition, the SalI fragment was released from pSPUTK vector with SalI and cloned into the SalI site of pMAL-c. There were some difficulties in subcloning T-250, T-335 and T-369 with the strategy of cloning BglII



Mutant	Amino acid sequence	Cloning Strategy	Constructed by
Sal	5-411	1	G. Werstuck*
T404	1-404	2	R. Wheatly*
T379	1-379	2	R. Wheatly*
T369	1-369	2	R. Wheatly*
T335	1-335	2	R. Wheatly*
T299	1-299	2	R. Wheatly*
T290	5-290	3	P. Shaw
T250	1-250	2	R. Wheatly*
T200	5-200	3	P. Shaw
T181	5-181	3	P. Shaw
T151	5-151	3	P. Shaw
T73	5-73	3	P. Shaw
$\Delta$ 25-141	5-25 141-411	4	G. Werstuck
$\Delta$ 141-178	5-141 178-411	4	G. Werstuck

**Table 5.1.1 Deletion mutations of Vmw65.** Shown here are some mutants constructed for studying the VIC assembly and other properties of Vmw65 involved in, including DNA binding, cooperative interaction with Oct-1 and interaction to VCAF-1 as well as interaction to VHS. Cloning strategies: 1, insertion of the Sal fragment into Sall site; 2, insertion of a termination codon into a desired site; 3, double digestion then ligation; 4, double digestion then ligation with linkers.

\* Subcloned into pRIT2T and pMAL-c2 by P. Shaw.

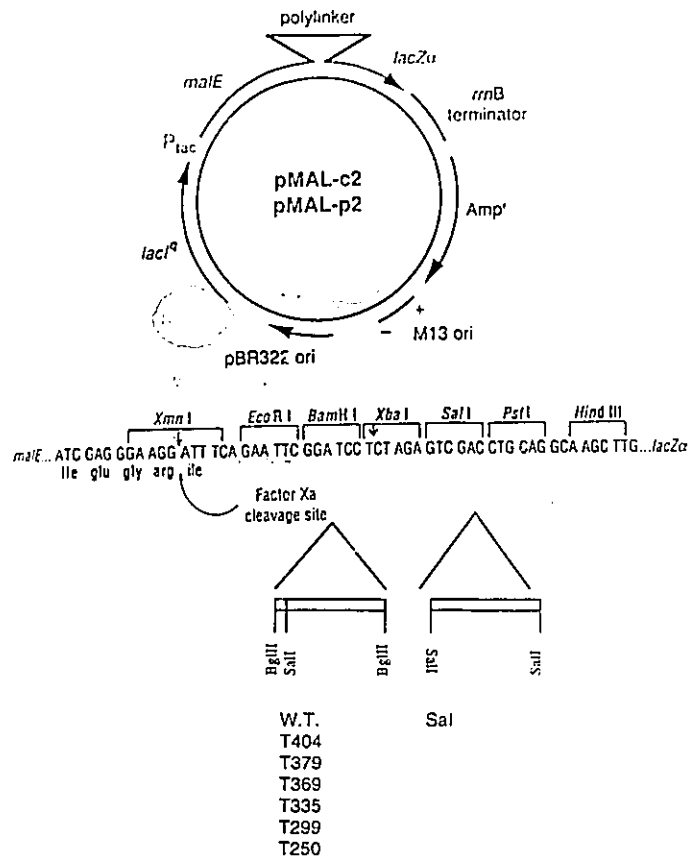
fragment into BamHI site. Therefore, the insert and the vector were first one-end blunted, then cloned into the BamHI site unidirectionally. These constructs (Figure 5.1.1) were transformed into DH-5 $\alpha$  bacterial strain and screened for the presence of insert and correct orientation.

The maltose binding protein and fusion proteins with Vmw65 and mutants (referred to as MBP, MBP-65, MBP-Sal, MBP-T250, MBP-T-299, MBP-T335, MBP-T369, MBP-T379, MBP-T404) were expressed and affinity purified using amylose resin as described in the Materials and Methods (Chapter 2). A final concentration of 0.3 mM IPTG was used to induce the expression of malE gene. The PD-10 column (Bio-Rad) was used to remove the maltose and change the protein into buffer D (Dignam et al., 1983). The protein amount was determined by using Bio-Rad assay, and the purity was examined by SDS-PAGE (Figure 5.1.2). It was found that the yield of MBP was higher than those of the fusion proteins, MBP alone gave 15 mg/500 ml culture, while the average yield for Vmw65 fusion proteins was 5 mg/500 ml culture.

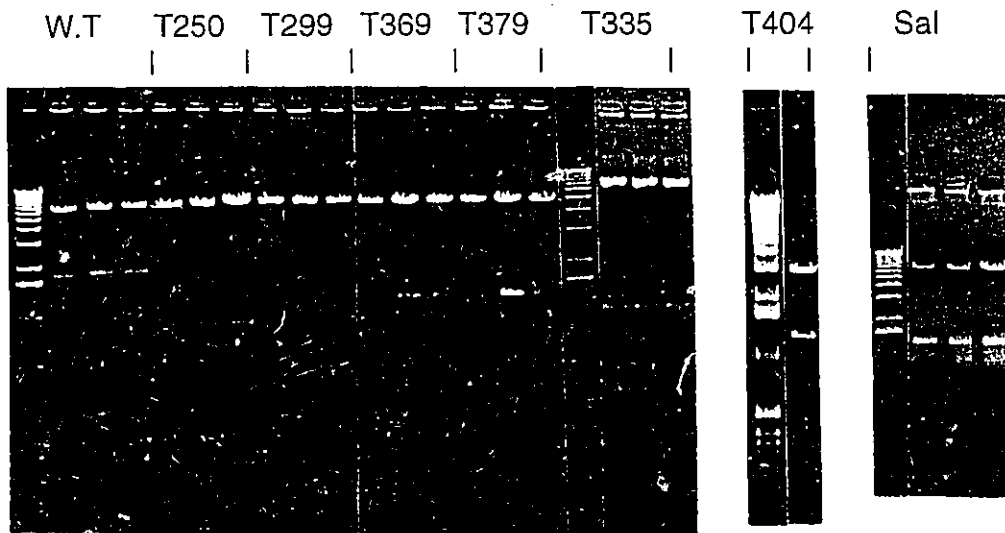
## 5.2 Results

### 5.2.1 VIC formation

To test the minimum requirement of Vmw65 for VIC formation, the MBP fusion proteins of Vmw65 and mutational derivatives were incubated with the TAATGAPAT DNA probe in the presence of GST-POU and purified VCAF-1 (figure 5.2.1). These

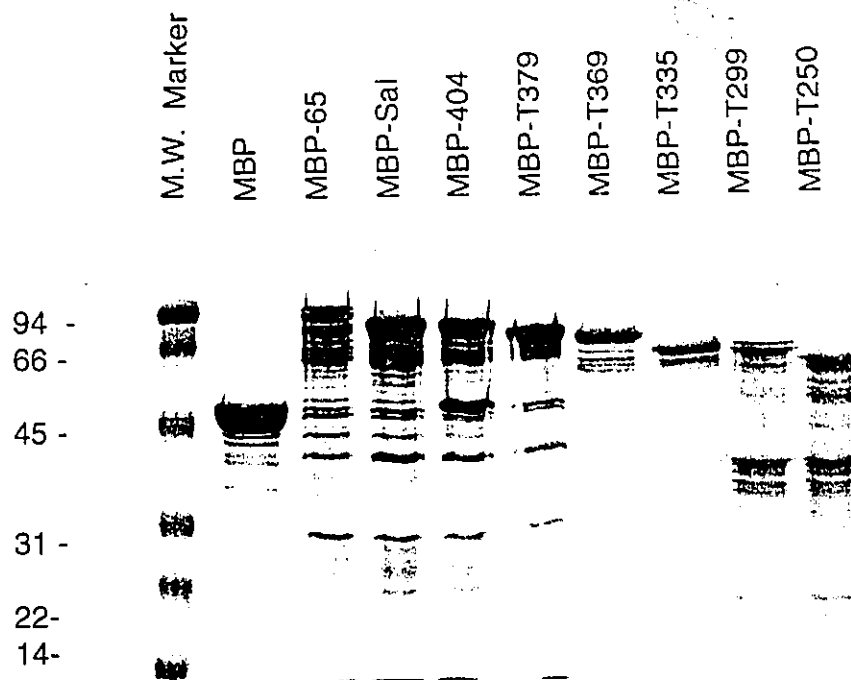


A



B

**Figure 5.1.1 Subcloning of Vmw65 and truncation mutants into pMAL-c2** Vmw65 and mutants were cloned into the BamHI site (except for the Sal fragment, which is cloned into SalI site) of *E. coli* expression vector pMAL-c2 (Panel A). The constructs were digested with Sal I to examine the presence of inserts (Panel B). The orientation of the inserts was checked by digesting with Sal I (for BamHI inserts) or KpnI and EcoRI (for SalI inserts) (data not shown).

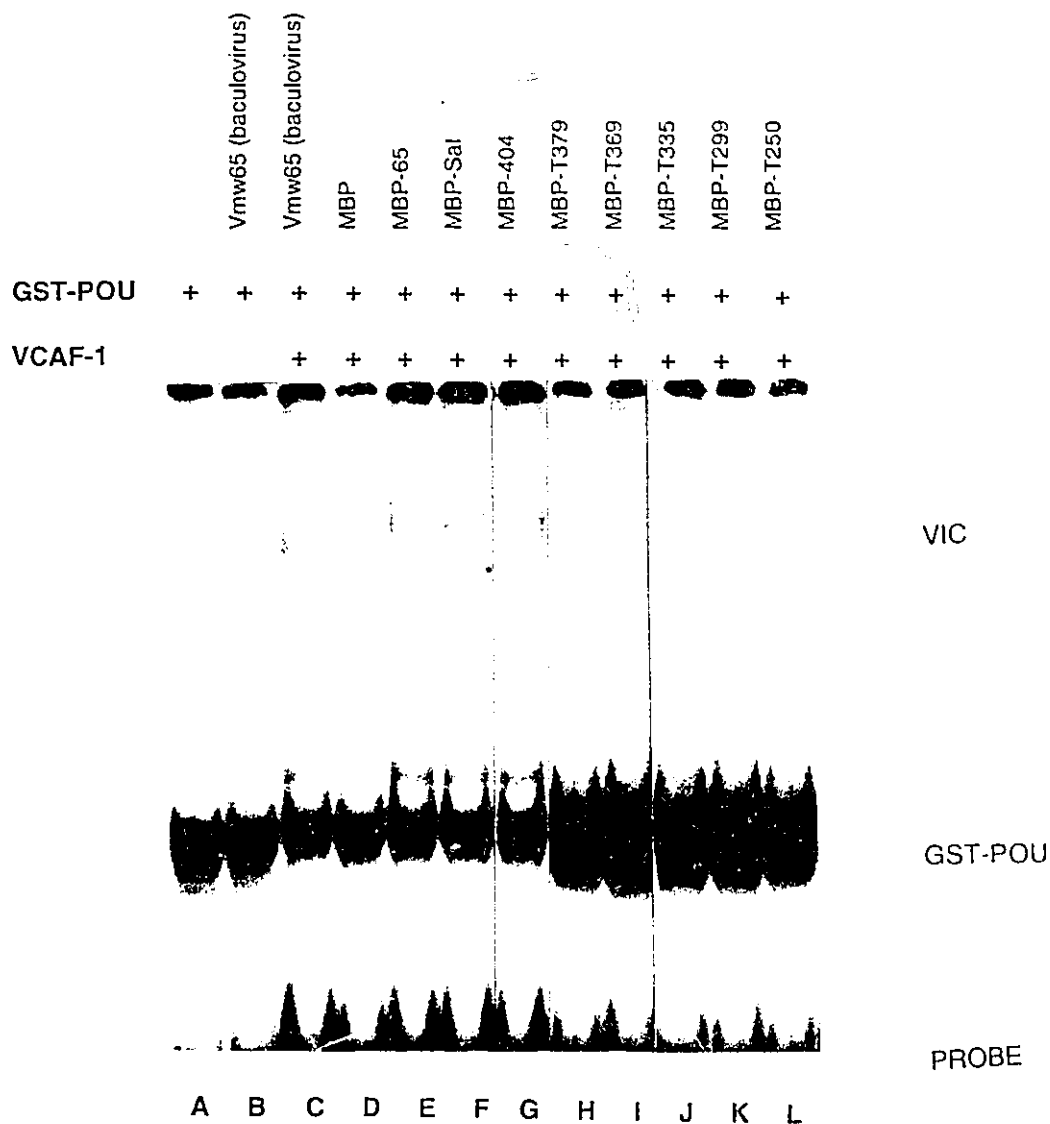


**Figure 5.1.2** Vmw65 and its mutants expressed as MBP fusion proteins using pMAL-c2 vector and purified by amylose resin. Shown here, purified MBP and Vmw65 fusion proteins were resolved a 10% SDS-PAGE gel. M.W. is the molecular weight standard; MBP is maltose binding protein; MBP-65, MBP-Sal, MBP-T404, MBP-T379, MBP-T369, MBP-T335 and MBP-T299 are MBP fused to full length Vmw65, Sal fragment and C-terminal truncation mutants T404, T379, T369, T335, T299, and T250 respectively.

experiments revealed that C-terminal truncation T404 was still able to form VIC but T379 and further removal of amino acids failed to generate the complex. The results were consistent with the previous findings (Greaves and O'Hare, 1989, 1990) that the carboxyl terminal boundary required for Vmw65 to mediate the assembly of VIC was located between amino acids 380-388.

### 5.2.2 Intrinsic DNA binding activity

Studies described in previous chapters had found that protein A fused Vmw65 had weak independent DNA binding activity when it was incubated with the TAATGARAT DNA probe at a high concentration. However, such intrinsic DNA binding activity was not observed with Vmw65 prepared from HSV-1 infected cell extract or with GST fused Vmw65. Whether it was a real property of Vmw65, or just an artifact due to the high concentration of the fusion proteins remains unclear. The DNA binding region of Vmw65 might be normally masked by the natural folded structure, and the interactions with DNA bound Oct-1 POU domain or VCAF-1 alters the conformation of Vmw65 so that the DNA binding region of Vmw65 can be exposed to the DNA target. To prove such an hypothesis, the independent DNA binding activity needs to be experimentally demonstrated as a true property of Vmw65. We also wanted to show whether the interaction of Vmw65 with other cellular factors would affect the affinity of Vmw65 to target DNA. The region of Vmw65 that



**Figure 5.2.1** VIC formation was examined with MBP-Vmw65 and mutants

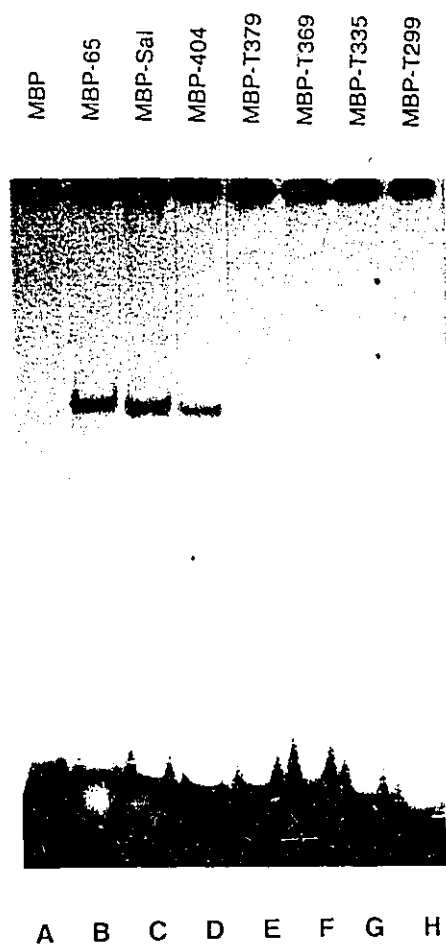
0.5  $\mu$ g of MBP and fusion proteins were incubated with the DNA probe in the presence of 0.05  $\mu$ g of GST-POU and 3  $\mu$ l of VCAF-1 and the complexes were resolved on a native gel. Shown here, lanes D to L are MBP, MBP-65, MBP-Sal, MBP-T404, MBP-T379, MBP-T369, MBP-T335, MBP-T299, and MBP-T250, respectively. Lanes A-C are controls containing GST-POU incubated alone (lane A) or in the presence of Vmw65 (lane B), or Vmw65 and VCAF-1 (lane C).

interacts with the DNA target should also be defined.

To examine the DNA binding activity of Vmw65, maltose binding protein fused to Vmw65 and its mutants were incubated with the TAATGARAT DNA probe in a mobility shift assay. The result showed that full length Vmw65, the Sal fragment and mutant T404 were able to bind to the DNA probe. This experiment provided evidence that Vmw65, when fused to another carrier protein, still possessed the independent DNA binding activity and the region up to amino acid 404 was required for this activity (Figure 5.2.2).

### 5.2.3 Cooperativity with DNA-bound POU-homeo domain of Oct-1

To map the region of Vmw65 that cooperatively interacts with POU domain of Oct-1 in the presence of the target DNA, mobility shift assays were carried out using MBP fusion proteins of Vmw65 and its mutants. As previously demonstrated by Stern and Herr (1991), and this lab, the POU domain of Oct-1 was sufficient to mediate a stable complex with Vmw65. When PA-POU was added to the reaction containing the DNA probe and MBP fused to Vmw65 (and mutant), the results (Figure 5.2.3) showed that the full length Vmw65 and the Sal fragment were able to efficiently interact with the DNA bound Oct-1 and generate a Vmw65-POU complex, while T404 was only capable of forming a weak complex. However, Vmw65 truncation to amino acid 379 failed to cooperatively interact with Oct-1.



**Figure 5.2.2** MBP-65, MBP-Sal and MBP-T404 were able to bind to DNA independently

8  $\mu$ g of MBP fusion proteins were incubated with the TAATGARAT probe. Shown here, lanes A to I are MBP, MBP-65, MBP-Sal, MBP-T404, MBP-T379, MBP-T369, MBP-T335, MBP-T299, and MBP-T250 respectively.

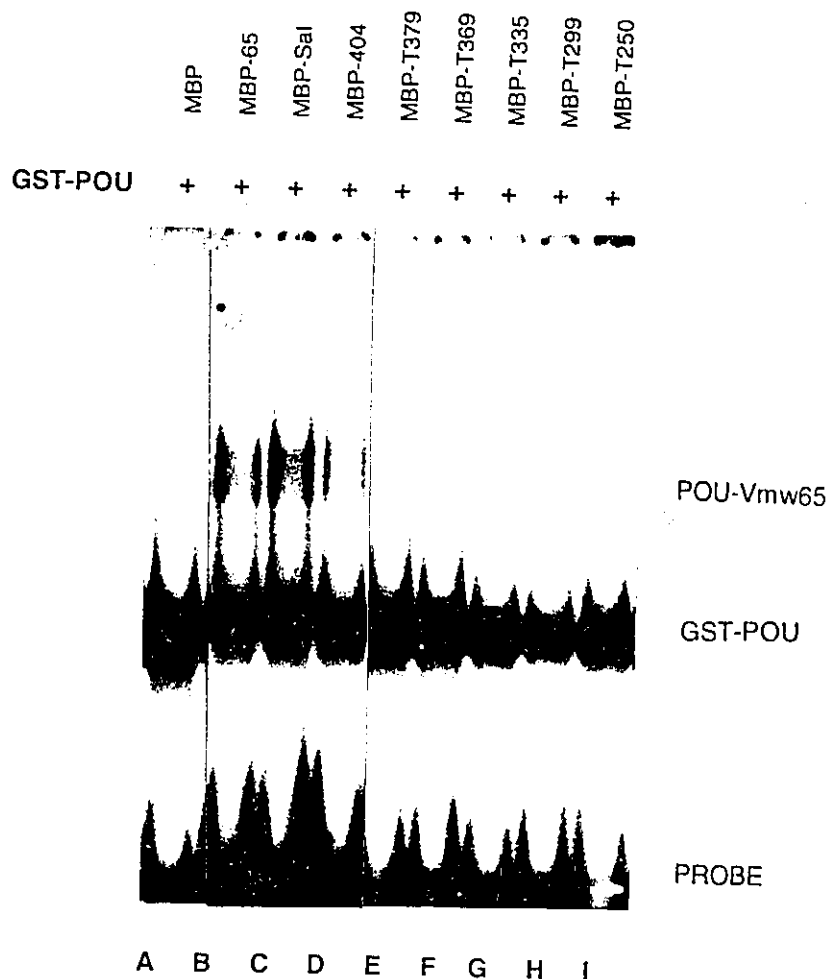


The results suggested that the amino acid residues from 379 downstream were involved in the interaction with POU domain of Oct-1 and DNA.

Because the formation of POU-Vmw65 complex exhibited the identical DNA specificity and the functional requirements to those of the VCAF-1 containing full complex (VIC) (Walker *et al.*, 1994), these residues (379-404) should be also important for the formation of Vmw65 induced complex. Our finding from this experiment supported the conclusion that the POU-Vmw65 complex and the VIC complex had the same domain requirement for Vmw65. If the POU-Vmw65 complex is the prerequisite for the subsequent assembly of the VIC complex, then, role of VCAF-1 should be to stabilize the full complex.

#### 5.2.4 Independent interaction with VCAF-1

Because VCAF-1 interacts directly with Vmw65, the investigation of interactions between Vmw65 and VCAF-1 is the key to understand the role of VCAF-1 in Vmw65 induced complex assembly. Mapping the region of Vmw65 that directly interacts with VCAF-1 would be an important step towards such understanding. The MBP fusion proteins of Vmw65 and its mutants were utilized as ligands in micro-affinity assays to locate the region of Vmw65 that is responsible for the VCAF-1 binding activity. MBP and fusion proteins of Vmw65 and its mutants were coupled onto the amylose resin at conditions determined by the previous pilot experiment (see materials and

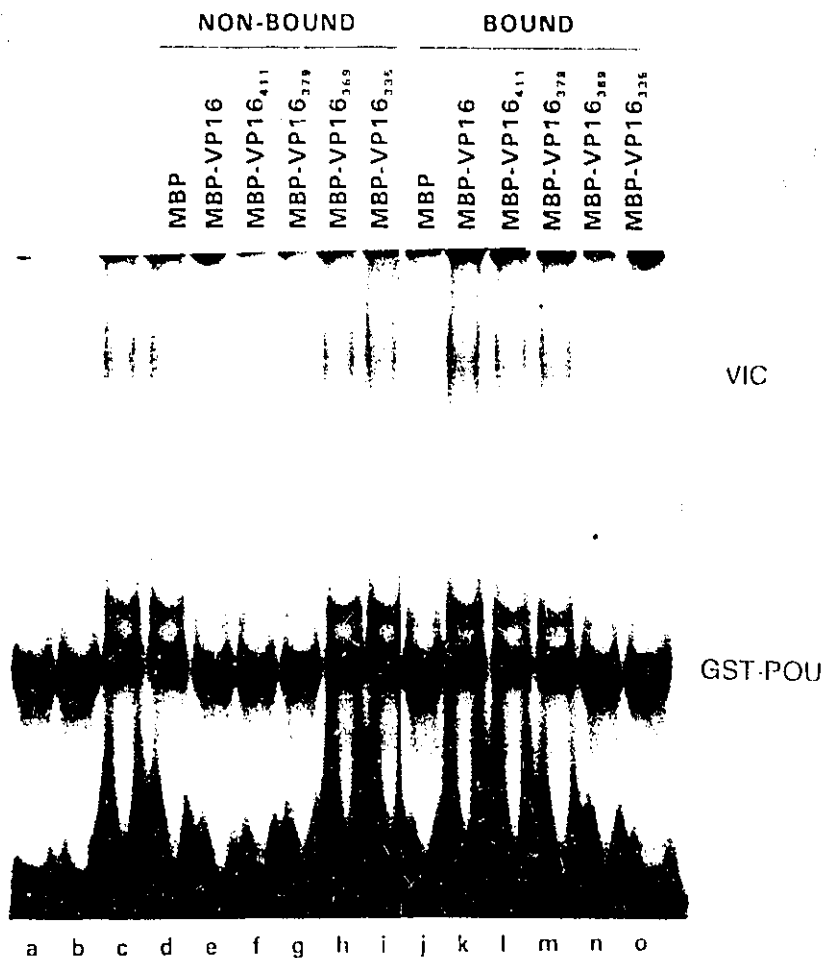


**Figure 5.2.3** MBP-65, MBP-Sal and MBP-T404 were able to cooperatively interact with DNA-bound POU domain

0.5  $\mu$ g of MBP fusion proteins were incubated with the TAATGARAT probe in the presence of 0.05  $\mu$ g of GST-POU. Shown here, lanes A to I are MBP, MBP-65, MBP-Sal, MBP-T404, MBP-T379, MBP-T369, MBP-T335, MBP-T299, and MBP-T250, respectively.

methods).

In order to examine the interaction between VCAF-1 and Vmw65 coupled beads, mobility shift assays were performed with the supernatant and eluent from micro-affinity assay. It was expected that the VCAF-1 activity would be depleted from the supernatant after incubating with the beads coupled with Vmw65 or mutant fusion proteins, to which VCAF-1 bound. Therefore, the VCAF-1 activity could be detected by the addition of the supernatant into the reaction mixture containing PA-POU, Vmw65 and the TAATGARAT DNA probe. As demonstrated in Figure 5.2.4, VCAF-1 activity was depleted from the supernatant of the affinity resin coupled with MBP-65, MBP-Sal, MBP-379, but not from those coupled with MBP-369, MBP-335, MBP-299 and MBP. When the bound material from the affinity resin were eluted, the VCAF-1 activity was recovered from MBP-65, MBP-Sal and MBP-379. The results indicate that amino acid residues from 369 to 379 are important for VCAF-1 binding and region 379-411 is required for VIC formation but not for independent interaction with VCAF-1. These results were consistent with our previous observation with linker insertion mutants, among which the linker insertion at position 369 abolished the VCAF-1 binding activity of Vmw65. More importantly, our findings with this experiment was the first indication that the determinants of VCAF-1 binding overlaps but are distinct from those of VIC formation.



**Figure 5.2.4 Binding of VCAF-1 to the truncated Vmw65**

Micro-affinity binding assays were performed as described, using MBP or the various MBP-Vmw65 fusion proteins immobilized on amylose resin. Bound and non-bound fractions were assayed for the VCAF-1 activity in the presence of 0.05  $\mu$ g of GST-POU and 2  $\mu$ l of VCAF-1 (lanes d to o). Lanes a-c are controls containing GST-POU incubated alone (lane a) or in the presence of Vmw65 (lane b), or Vmw65 and VCAF-1 (lane c).

Figure 5.2.4

### 5.3 Mapping the region of Vmw65 required for VIC formation using synthetic peptides

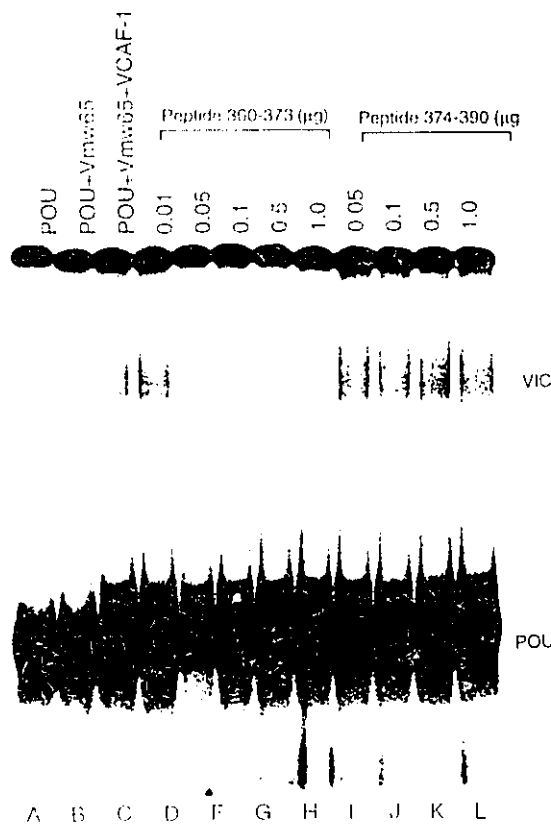
Using synthetic peptides, Hiagh et al., (1990) showed that the ability of Vmw65 to mediate VIC formation was disrupted by the peptide containing Vmw65 amino acid residues 360-373. Hayes and O'Hare (1993) further speculated that peptide 360-367 might directly interact with the cellular factor CFF and block VIC formation. However, they did not test the effect of synthetic peptides on VIC assembly with purified components. To determine whether the disruption of VIC formation by synthetic peptides was related to VCAF-1 binding, two peptides containing amino acids 360-373 and 374-390 (provided by Dr. Hensley, SmithKline Beecham Pharmaceutical) were utilized. If the synthetic peptide represents the VCAF-1 binding region of Vmw65, it should bind to VCAF-1 and occupy the Vmw65 interacting surface of VCAF-1. Therefore, when excess amount of the peptide is preincubated with VCAF-1 prior to the addition of Vmw65, the interaction between Vmw65 and VCAF-1 should be displaced by the peptide and result in the inhibition of VIC formation. By comparison of the results of peptide inhibition with micro-affinity VCAF-1 binding experiments, the Vmw65 and VCAF-1 interaction could be further defined.

The sequences of the two peptides used are:

Vmw65<sub>360-373</sub>: R-E-H-A-Y-S-R-A-R-T-K-N-N-Y

Vmw65<sub>374-390</sub>: G-S-T-I-E-G-L-L-D-L-P-D-D-D-A-P-E

Mobility shift assay was performed using the following procedure to map the region(s) of Vmw65 involved in VIC assembly. The peptides (from 0.001 to 1  $\mu\text{g}$ /reaction) were preincubated with purified VCAF-1 and POU domain of Oct-1 at room temperature for 15 minutes, then the viral Vmw65 and radiolabelled TAATGARAF DNA probe were added and incubated at 30°C for another 20 minutes. The reaction mixture was run on a mobility shift gel as described previously. Another incubation without peptide was used as a control. The mobility shift analysis showed that the synthetic peptide containing Vmw65 360-373 inhibited the formation of VIC when used in excess 0.1  $\mu\text{g}$ . However, peptide 374-390 could not inhibit the formation of VIC even with amounts over 5  $\mu\text{g}$  (Figure 5.3.1). The interpretation of these results was that the region of Vmw65 that interacts with VCAF-1 probably lies between amino acids 360-373. As a result, it occupies the space of VCAF-1 which normally interacts with Vmw65. When present in excessive amount, the peptide Vmw65<sub>360-373</sub> saturates the Vmw65 binding site of VCAF-1 to prevent the further interaction between VCAF-1 and Vmw65 resulting in the blocking of VIC assembly. That the peptide Vmw65<sub>374-390</sub> had no effect on VIC assembly indicated that this region probably did not interact with VCAF-1 directly, although it is required for the cooperative interaction with DNA bound Oct-1. In the previous section, the micro-affinity assays with Vmw65 truncation mutants had demonstrated that the removal of amino acids 379-411 abolished



**Figure 5.3.1 Mapping the region of Vmw65 that interacts with VCAF-1 using peptide competition experiment**

Titration amount of the synthetic peptides Vmw65 360-373 (lanes D-H containing 0.01, 0.05, 0.1, 0.5 and 1  $\mu\text{g}$ , respectively) and Vmw65 374-390 (lanes I-L containing 0.05, 0.1, 0.5 and 1  $\mu\text{g}$ , respectively) were incubated with 0.05  $\mu\text{g}$  of GST-POU and 2  $\mu\text{l}$  of VCAF-1 at room temperature for 15' prior to the addition of viral Vmw65 and the DNA probe. The reactions were incubated at 30  $^{\circ}\text{C}$  for another 20' and resolved on a band shift gel. Lanes A-C are controls containing GST-POU incubated alone (lane A) or in the presence of Vmw65 (lane B), or Vmw65 and VCAF-1 (lane C).

VIC formation but kept VCAF-1 binding intact. However, when Vmw65 was further truncated to 369, it could not independently bind to VCAF-1. Therefore the region around 370 is critical for VCAF-1 binding.

#### 5.4 Summary of part 1

Several carboxyl terminal truncation mutants of Vmw65 have been constructed and expressed as fusion proteins of maltose binding protein. Using the purified *E. coli* expressed MBP-fusion proteins of these mutants, various properties of Vmw65 involved in the assembly of multi-component DNA binding complex have been examined. Mobility shift assays, together with the micro-affinity assay and synthetic peptide competition experiments, were carried out to probe the requirement of Vmw65 for each of the distinct protein-protein and protein-DNA interaction involved in the assembly of multi-component DNA binding complex. Figure 5.4.1 summaries the results of examining the individual interactions, including the intrinsic weak DNA binding of Vmw65, the cooperative DNA binding with the POU domain of Oct-1, and the independent association with VCAF-1.

Previous studies had shown that Vmw65 had weak sequence-specific DNA binding activity at high protein concentration (Kristie and Sharp, 1990; Stern and Herr, 1991; and experiment described in chapter 4). However, the putative DNA binding domain of Vmw65 had not yet been determined. Stern





Figure 5.4.1 Summary of mutational analysis with truncated Vmw65.

and Herr (1991) showed that the synthetic peptide containing amino acids 170-202 of Vmw65 was able to bind to DNA without sequence-specificity. Our study has demonstrated that Vmw65 truncated up to amino acid 404 was still capable of binding to DNA with lower affinity than those of wild type and Sal fragment. However, further removal of an additional 25 amino acids (T379) totally abolished the intrinsic DNA binding activity of Vmw65. This result indicates that the region between 379 and 404 is required for Vmw65 to bind to DNA. Our previous study using insertion mutants has shown that linker insertions in amino acids 178 and 241 also disrupt the DNA binding activity but insertion at 369 still weakly binds to DNA (Chapter 4). Therefore, the requirement for DNA binding may be separated into two subregions, one containing residues from 379 downstream, the other in consistent with the region 1 previously defined by Stern and Herr (1991). Our findings shows neither region alone is sufficient for the intrinsic DNA binding activity of Vmw65.

The putative region required for cooperative interaction with Oct-1 had previously been localized to amino acids 378-389 (Stern and Herr, 1991). This study found that the cooperative Oct-1 binding activity almost corresponded to the intrinsic DNA binding activity and the region containing amino acids 379-404 was essential for the cooperativity. Latter study by Hayes and O'Hare (1993) using synthetic peptide competition also indicated that the residues between

375 and 385 might be involved in the interaction with Oct-1.

The requirement for Vmw65 to interact with VCAF-1 was investigated using micro-affinity assays with Vmw65 truncational derivatives and synthetic peptide competition. The removal of amino acids 369-379 abolished the independent VCAF-1 binding activity of Vmw65. We also found that synthetic peptide containing residues 360-373 inhibited VIC formation when preincubated with VCAF-1. These findings demonstrated that amino acid residues around position 370 were essential for the independent interaction of Vmw65 with VCAF-1. This region was also believed to have a surface-exposed conformation since it was sensitive to protease digestion (Hayes and O'Hare, 1993).

Interestingly, although the requirement for Vmw65 to cooperatively interact with DNA-bound Oct-1 seems to correlate with VIC assembly (this thesis; Walker et al., 1994), the independent interaction of Vmw65 to VCAF-1 exhibits distinct requirements to that of VIC formation. Mutant T379 can still bind to VCAF-1 independently but fails to mediate VIC assembly. Therefore, the prior interaction between Vmw65 and VCAF-1 may be necessary but is not sufficient for mediating the formation of the full complex. This indicates the additional level of protein-protein interactions involved in VIC assembly.

Deletion mutants may cause artificial conformational change because of the removal of large segments from the

protein. This would influence some of our results when these mutant were used to investigate requirements for VIC assembly. Furthermore, the activation function of complexes generated with these mutants could not be examined due to the lack of an activation domain. Therefore, in order to further investigate the requirements of these individual interactions involved in VIC assembly and their relevance to the activation of IE genes, site-directed mutations of some selected amino acids within these two regions of Vmw65 were chosen to be the next step of this project.

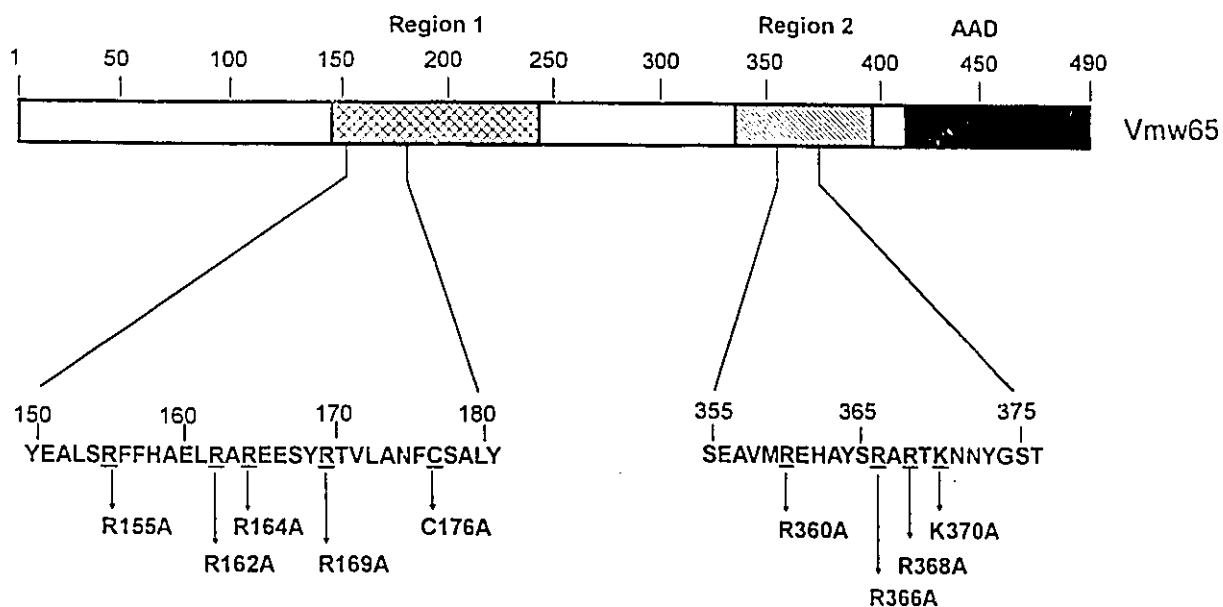
## Part 2. Site-directed mutagenesis

The use of large deletions or linker insertions might interrupt the natural secondary structure of Vmw65 and cause the conformational changes that would indirectly affect complex formation and individual protein-protein interactions. The substitution of charged amino acids by alanine residues had been proved to be an effective method to probe the protein-protein interactions of cloned proteins while causing only minor perturbation in protein structure (Gibbs and Zoller, 1991). At the time I started this project, the only reported site-directed mutagenesis study on Vmw65 (Greaves and O'Hare; 1990) had shown that amino acid substitutions within the region of residues 364-380 could affect the VIC formation and transactivation. However, the influence of the site-specific mutations on individual protein-protein and protein-DNA interactions had not been tested.

Previous mutagenesis studies of Vmw65 described in this thesis and by others (Ace et al., 1988; Westuck and Capone, 1989 a,b; Greaves and O'Hare, 1990; Stern and Herr, 1991, Hayes and O'Hare, 1993) has revealed that two subregions within the amino terminal 411 residues are important for the assembly of the transcriptional active Vmw65 multi-component complex. These are referred to as region 1, containing amino acid residues 141-250, and region 2, spanning residues 335-390. Several properties of Vmw65 involved in the assembly of VIC, including intrinsic DNA binding, cooperative interaction

with the POU domain, independent VCAF-1 binding, and the recently defined interaction with VHS (Smibert et al., 1994), require both regions. These regions have predicated amphiphathic  $\alpha$ -helical structures, and are rich in positively charged amino acids, particularly in arginines.

In order to investigate the requirements of individual protein-protein interactions associated with Vmw65 as well as their roles in the assembly of a transcriptionally active complex, we decided to selectively substitute some positively charged amino acid residues in both regions 1 and 2 by oligonucleotide directed mutagenesis. Because positively charged residues are often involved in both protein-protein and protein-DNA interactions, the replacement of these amino acids by alanines might be expected to disrupt some distinct interactions. Alanine is a neutral amino acid and usually does not change the conformation of the peptide backbone (Chou and Fasman, 1978). Therefore, it was chosen as a substitute for selected residues within regions 1 and 2 (Figure 5.5.1). The substituted residues included arginines at codons 155, 162, 164, 169, 360, 366 and 369. In addition, a cysteine residue at 176 was replaced by alanine to examine the role of the sulfhydryl group. The lysine residue at position 379 was also substituted since the region surrounding it had been reported to be exposed on the surface and important for VIC formation (Hayes and O'Hare, 1993).



**Figure 5.5.1 Schematic representation of Vmw65**

The amino acid sequence is indicated by the numbers on the top of the bar. The filled box represents the acidic activation domain; both regions 1 and 2 are indicated with hatched boxes. The sites where the charged amino acids were substituted by alanine are indicated by single letter codes. The nomenclature refers the alteration of amino acid residue and its position in the primary sequence.

## **5.5 Results of oligonucleotide directed mutagenesis**

Oligonucleotide directed mutagenesis was carried out as described in Materials and Methods (Chapter 2). The results showed that 75% of the plaques contain the mutations introduced by mutagenic oligonucleotides (Figure 5.5.2).

Vmw65 and the various mutant derivatives were cloned into the *E. coli* expression vector, pMAL-c2, and expressed as fusion proteins of maltose binding protein (MBP). These constructs (referred to as pMAL-Sal, pMAL-R155A, pMAL-R162A, pMAL-R164A, pMAL-R169A, pMAL-C176A, pMAL-R360A, pMAL-R366A, pMAL-R368A and pMAL-K370A), which all encoded residues 4 to 411 without the acidic activation domain, were purified by affinity chromatography on amylose resin as described in chapter 2. The products were examined on an SDS-PAGE electrophoresis gel. The results showed that all Vmw65 derivatives generated by oligonucleotide site-directed mutagenesis have the same size as their wild type counterpart (MBP-Sal) (Figure 5.5.3).

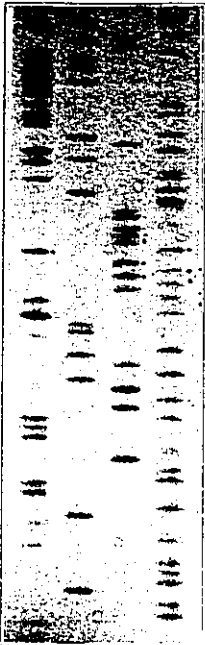
## **5.6 In vitro Analysis of site-directed mutants**

### **5.6.1 Intrinsic DNA binding activity of Vmw65**

Our previous results using carboxyl terminal truncation mutants showed that Sal fragment fused to Protein A and MBP possessed the weak intrinsic DNA binding activity to the TAATGARAT probe when used at a concentration approximately 20 times higher than that required for VIC formation. It was

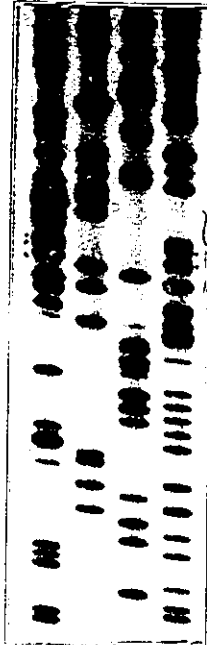


VP16 R155A



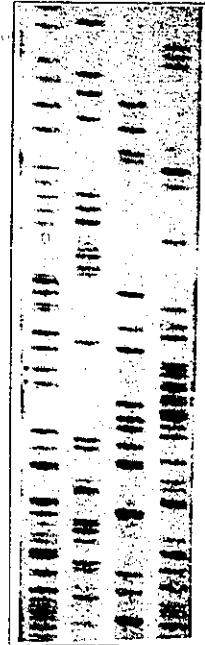
G A T C

VP16 R162A



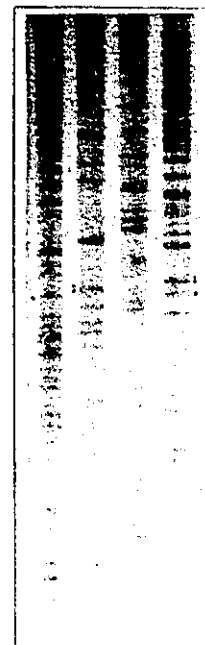
G A T C

VP16 R164A



G A T C

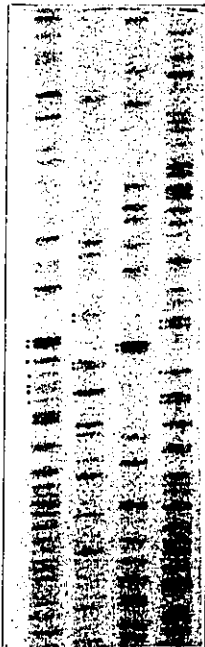
VP16 R169A



G A T C

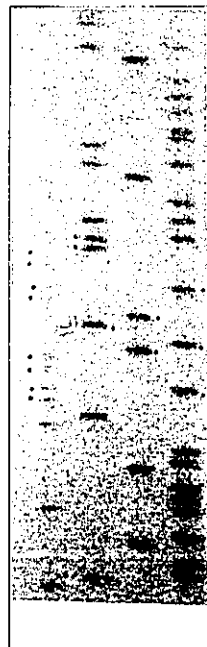
174

VP16 C176A



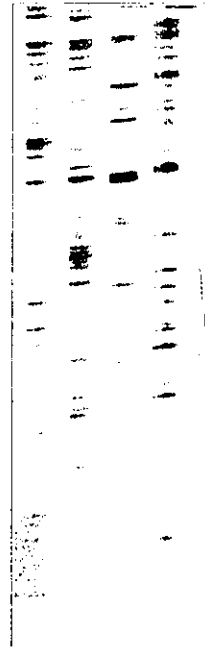
G A T C

VP16 R360A



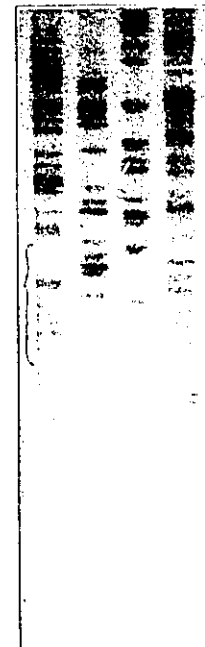
G A T C

VP16 R368A



G A T C

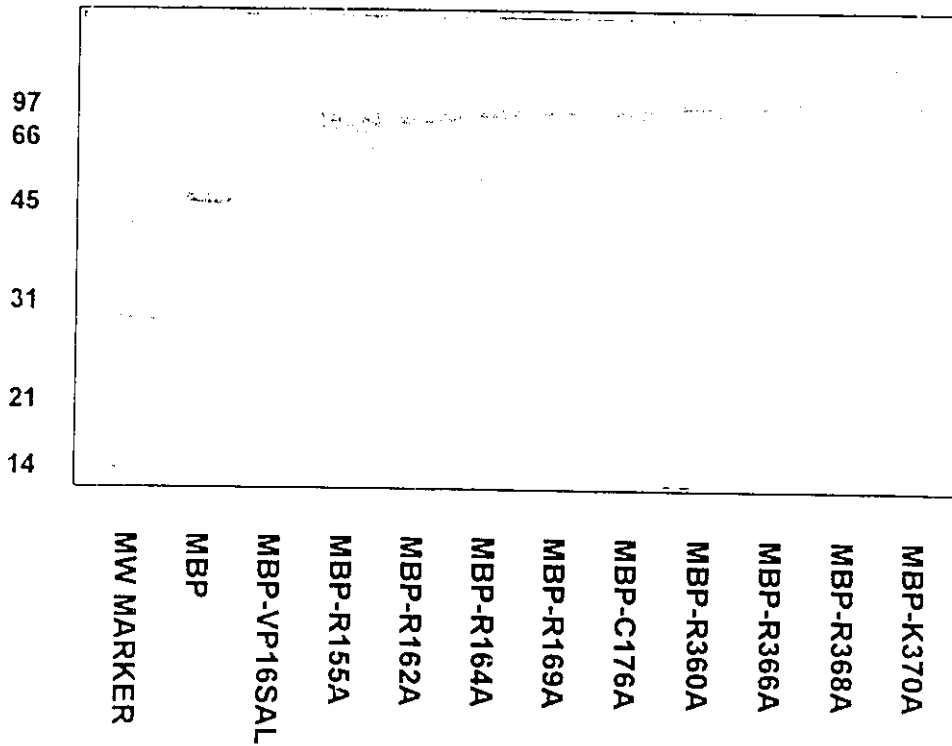
VP16 K370A



G A T C

Figure 5.5.2 DNA sequencing analysis of mutations

Shown here, the sequencing gels demonstrated that the point mutations had been introduced into the Vmw65 gene. The arrows indicate where the mutagenic sequences are.



**Figure 5.5.3 Vmw65 and its mutants expressed as MBP fusion proteins**

Shown here, the wild type and mutant Vmw65 MBP fusion proteins were purified by amylose resin and resolved on an SDS-PAGE gel. The molecular size markers, MBP, and fusion proteins are indicated at the bottom of the figure.

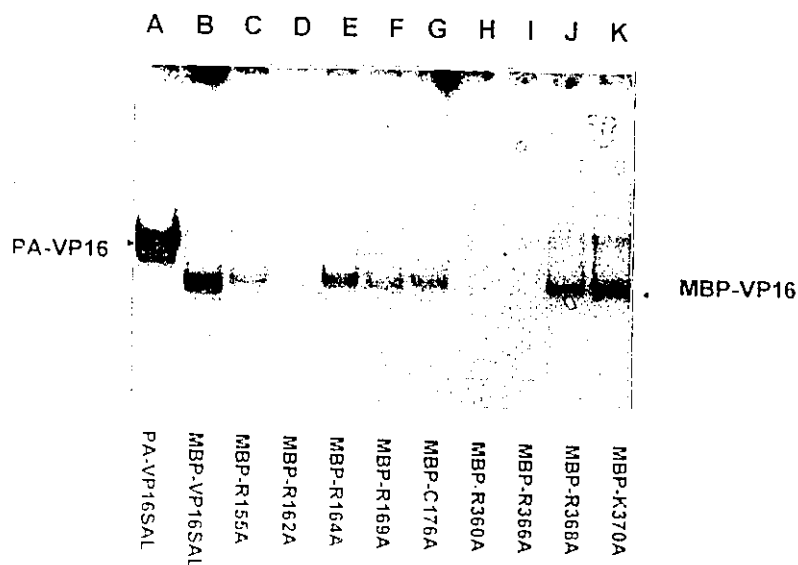
further demonstrated that deletion from 404 to 379 abolished this activity. To investigate this property of Vmw65 in detail, the Sal fragment containing single amino acid substitutions were purified as MBP fusion proteins and used to examine the intrinsic DNA binding activities at different concentrations. The *in vitro* DNA binding assay (Figure 5.6.1) showed that the MBP fused wild type Sal fragment was able to bind to the TAATGARAT probe from ICPO proximal region when used at a concentration 10 to 20 fold in excess to that required for VIC formation (lane B, in which 10  $\mu$ g of protein was used). As a control the PA-Sal was also incubated with the DNA probe and it generated a complex migrating slightly slower than MBP-Sal as expected (lane A). The protein A-Sal also exhibited stronger DNA binding activity. The mutants R155A, R164A, R169A, C176A, R368A and K370A were still able to bind to the DNA probe efficiently (lanes C, E, F, G, J and K, respectively). However, the replacement of arginine residues at 162, 360 and 366 with alanines abolished the DNA binding activity of the Sal fragment (lanes D, H and I, respectively). These results indicated that arginine residues 162, 366 and 368 might be directly involved in the interaction with DNA or contribute to the structure which is necessary for DNA binding. Interestingly, these residues are distributed in both subregions.

It had been demonstrated that the intrinsic DNA binding property of Vmw65 was sequence-specific and required the

presence of the downstream GARAT motif (Kristie and Sharp, 1990; Stern and Herr, 1991). To further examine whether this property and its requirement would also require the upstream ATGC motif, the mutant derivatives of Vmw65 were incubated with the (OCT-)TAATGARAT probe derived from ICP4 promoter region, in which the octamer element is compromised. A similar pattern of DNA binding was observed with these Vmw65 mutant derivatives (Figure 5.6.2). This result indicates that the upstream ATGC sequence is not necessary for the intrinsic DNA binding activity of Vmw65.

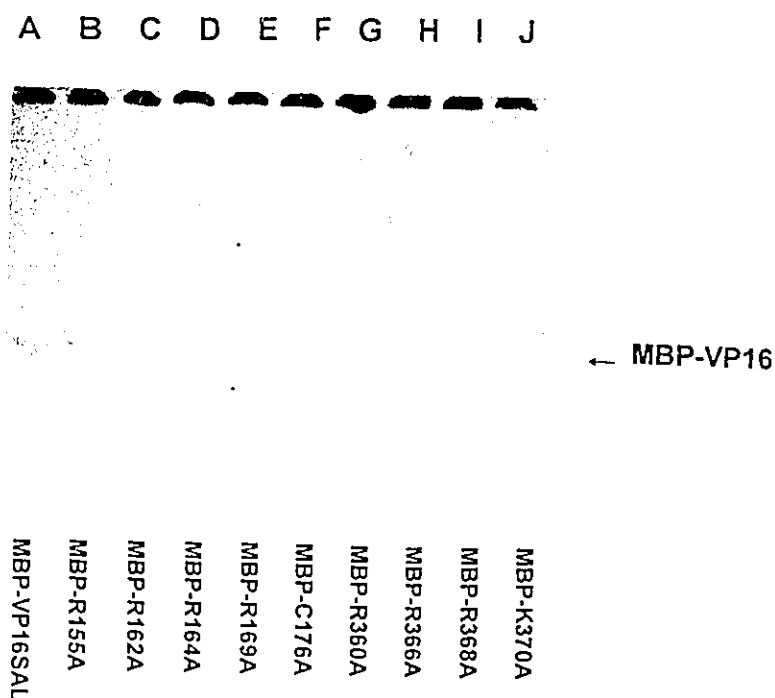
#### 5.6.2 The cooperativity with Oct-1 in DNA binding

To investigate cooperative interaction with DNA-bound Oct-1, the Vmw65 derivatives were incubated with GST-POU in reactions containing (OCTA+) and (OCTA-) DNA probe. As shown in Figure 5.6.3, when the POU domain of Oct-1 (GST-POU) was added to the incubation mixture containing MBP fused wild type or mutant Vmw65 Sal fragment and DNA probe, in addition to the formation of a specific POU-homeo domain DNA binding complex, another specific complex that migrated slightly faster than the Vmw65-DNA complex was observed on the mobility shift gel. The reason that this complex migrates faster than the Vmw65-DNA complex is not clear. It is possible that the particular conformation of the ternary complex confers faster electrophoretic mobility. Previous results obtained with PA-Sal also showed that the Vmw65-POU-DNA complex had the same as



**Figure 5.6.1** The intrinsic DNA binding activities of Vmw65 and mutants

MBP-fused Vmw65 and mutant derivatives (10  $\mu$ g) were incubated with the (OCTA+)TAATGARAT DNA probe and the protein-DNA complexes were resolved on a native gel. Shown here, lane A contained PA-Sal, which forms a slower migrating protein-DNA complex due to the larger size; lanes B to K contained MBP fused wild type Sal fragment or mutant derivatives as indicated. R162A, R360A and R366A are defective in DNA binding activity.



**Figure 5.6.2** The DNA binding activities of Vmw65 and mutants to the OCTA- probe

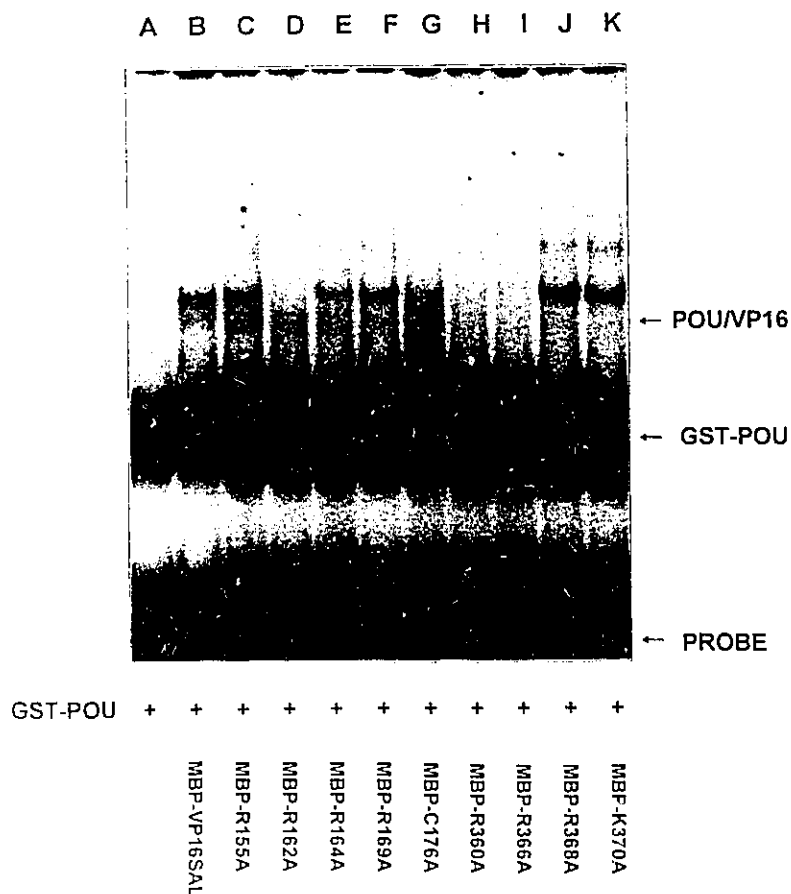
MBP-fused Vmw65 and mutant derivatives (10  $\mu$ g) were incubated with the (OCTA-)TAATGARAT DNA probe and the protein-DNA complexes were resolved on a native gel. Shown here, lanes A to J contained MBP fused wild type Sal fragment or mutant derivatives as indicated. The mobility shift gel shows the same profile as figure 5.6.1, in which the OCTA+ probe was used.

the mobility with Vmw65-DNA complex (Chapter 4). This specific DNA binding complex (POU-Vmw65) was generated with the wild type Vmw65 Sal fragment as well as mutants R155A, R162A, R164A, R169A and C176A (lanes B to G). All these residues are located within region 1; therefore, the substitution of arginine with alanine within region 1 has no effect on cooperativity with Oct-1 and DNA indicating that this region is probably not required for the formation of the Vmw65-Oct-1-DNA complex. It should be mentioned that mutant R162A abolishes the intrinsic DNA binding activity of Vmw65, but it can cooperatively bind to DNA in the presence of Oct-1. In contrast, mutants R360A, R366A, R368A and K370A were defective in cooperative DNA binding with Oct-1 (lanes H to K), although R368A and K370A were able to bind to DNA by their own. The interpretation of these results might be that the cooperativity between Vmw65 and DNA-bound Oct-1 is mediated by a protein-protein interaction through contacting region 2 of Vmw65 (at least from amino acid 360 downstream). The intrinsic DNA binding activity of Vmw65 is not a necessary prerequisite for the cooperative interaction with DNA-bound Oct-1.

Similar results were observed with the (OCTA-) TAATGARAT probe (Figure 5.6.4).

### 5.6.3. Direct interactions of Vmw65 mutants with VCAF-1

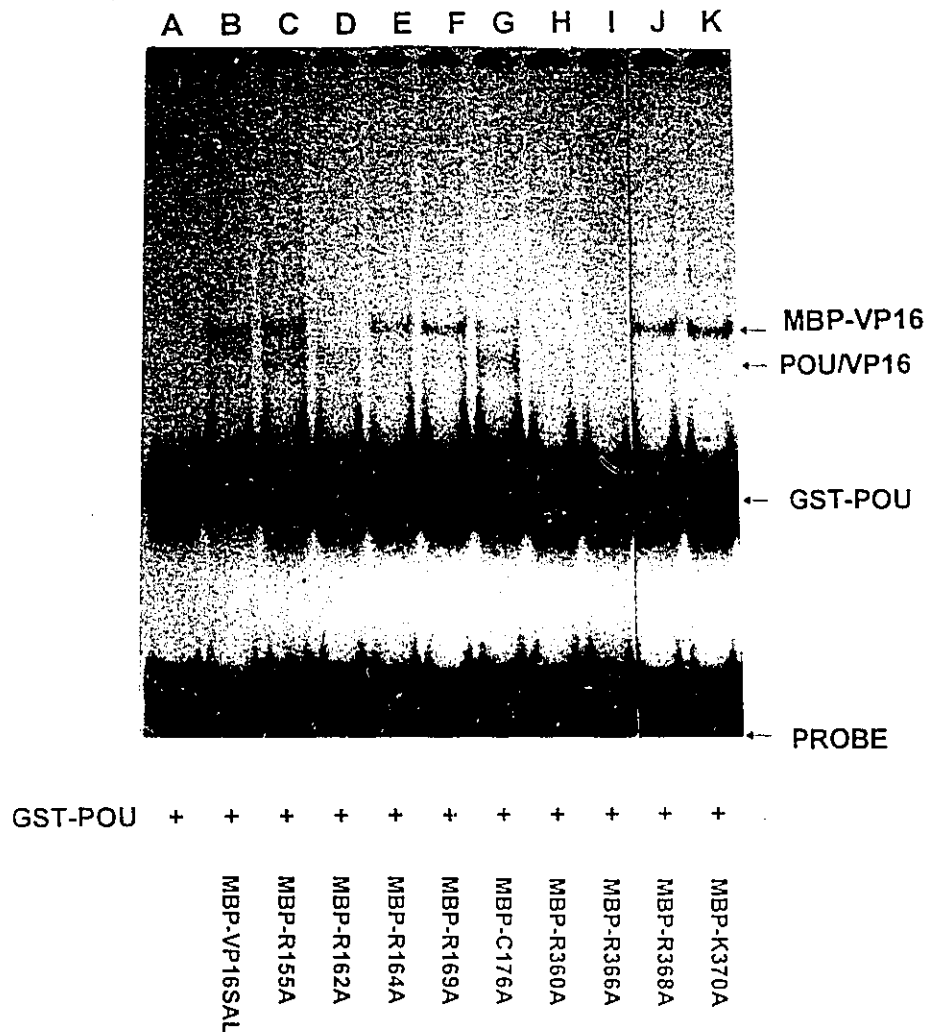
Direct VCAF-1 binding activity of Vmw65 derivatives were examined by micro-affinity assays as before. MBP-Sal and



**Figure 5.6.3 The cooperative interaction of Vmw65 with DNA bound POU domain**

The wild type and mutant Vmw65-MBP fusion proteins (6  $\mu$ g) were incubated with the (OCTA+)TAATGARAT DNA probe in the presence of 0.05  $\mu$ g of GST-POU. Lane A is GST-POU alone. The fusion proteins are indicated at the bottom of the gel (lanes B to K). The positions of protein-DNA complexes are indicated.





**Figure 5.6.4** The cooperative interaction of Vmw65 with DNA bound POU domain was analyzed with OCTA- probe

The wild type and mutant Vmw65-MBP fusion proteins (6  $\mu$ g) were incubated with the (OCTA-)TAATGARAT DNA probe in the presence of 0.05  $\mu$ g of GST-POU. Lane A is GST-POU alone. The fusion proteins are indicated at the bottom of the gel (lanes B to K). The positions of protein-DNA complexes are indicated. The mobility shift gel shows the same profile as figure 5.6.3, in which the OCTA+ probe was used.

other Vmw65 mutant derivatives were coupled onto the amylose resin under conditions determined by previous pilot experiments. As a control, MBP without fusion protein was also coupled onto the resin under the same conditions. Affinity purified VCAF-1 was incubated with the resin of various Vmw65 mutants. Mobility shift assay was performed with the bound and non-bound material collected from above micro-affinity assays to define the interactions of VCAF-1 with Vmw65 and its mutant derivatives. The presence of VCAF-1 in each fraction was determined by the VIC forming activity of the fraction in reactions containing GST-POU, wild type MBP-Vmw65 and DNA probe. VCAF-1 activity was depleted from the supernatant of MBP-Sal, MBP-R155A, MBP-R162A, MBP-R164A, MBP-C176A and MBP-R360A but not from MBP-R169A, MBP-R366A, MBP-R368A and MBP-K370A (figure 5.6.5, A). When the bound material on these beads were eluted, VCAF-1 activity was recovered from MBP-Sal, MBP-R155A, MBP-R162A, MBP-R164A, MBP-C176A and MBP-R360A (Figure 5.6.5, B). The findings that single amino acid substitutions at residues 169, 366, 368 and 370 disrupted the association of the Sal fragment with VCAF-1 indicated that these residues were critical for VCAF-1 binding, and moreover, that both region 1 and 2 were involved in the interaction between Vmw65 and VCAF-1. These results were consistent with the previous conclusions from C-terminal truncation and linker insertion mutagenesis, which showed that region 2 was important for VCAF-1 binding activity of Vmw65 since the



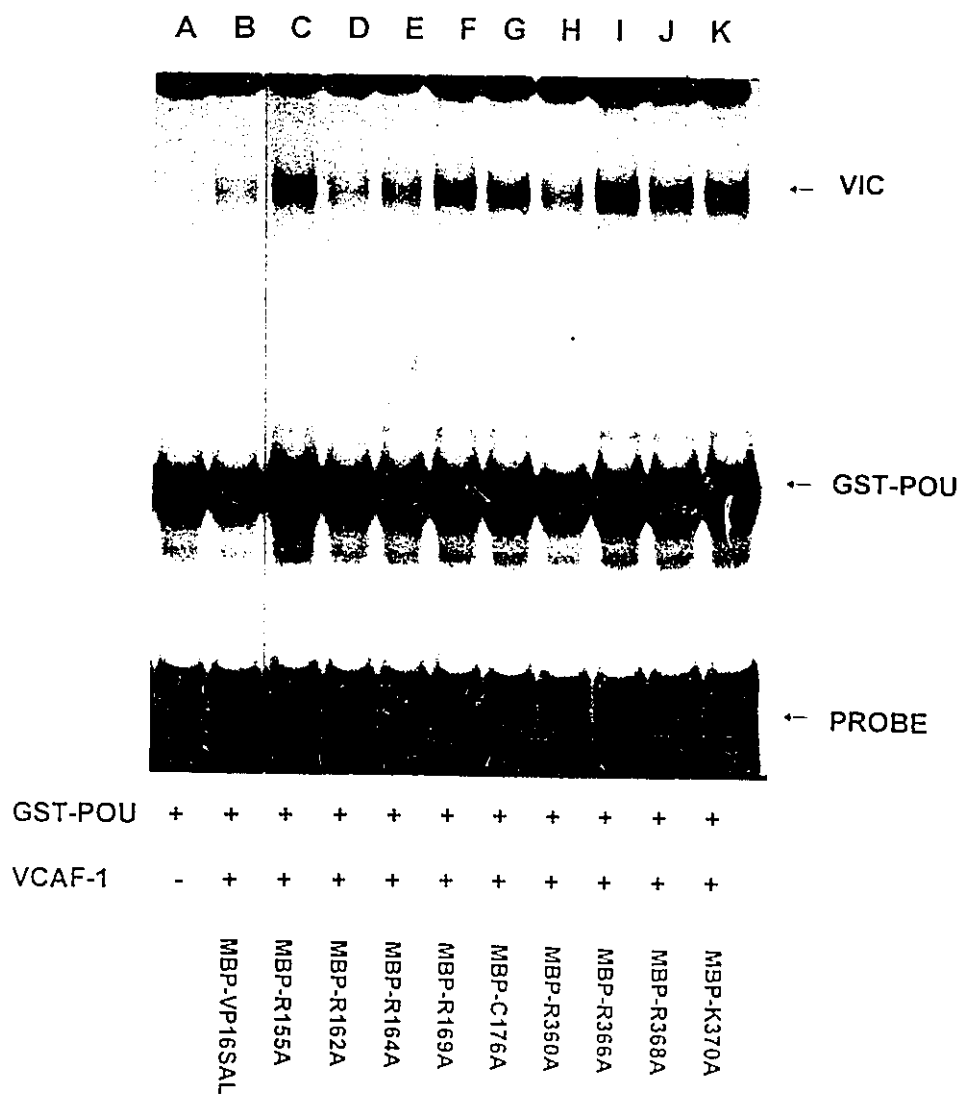
removal of residues 379 to 369 and the linker insertion at 369 abolished the VCAF-1 binding activity.

#### **5.6.4 Examination of the VIC assembly with POU domain, VCAF-1 and MBP-Vmw65 mutants**

The influence of the charge-to-alanine substitutions on VIC formation was examined by mobility shift assays using the mutants MBP fusion proteins. Surprisingly, all of the mutants were able to efficiently direct the formation of VIC (Figure 5.6.6). MBP-Sal and mutant derivatives were also capable of forming VIC with (OCTA-)TAATGARAT probe in the presence of GST-POU and VCAF-1 (Figure 5.6.7).

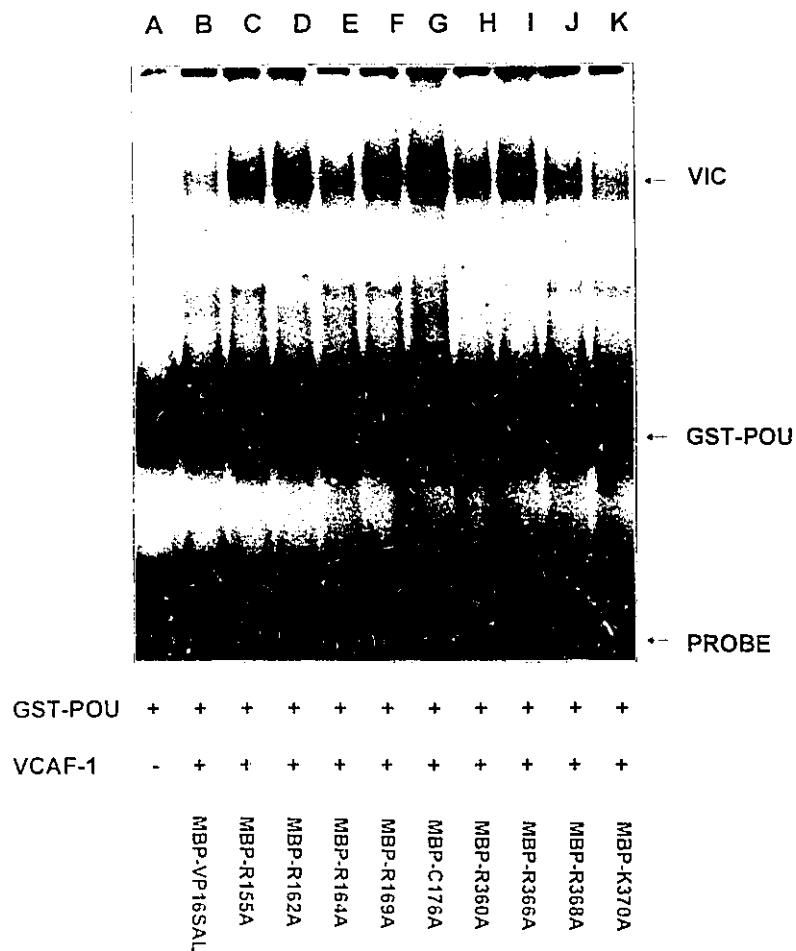
The fact that some mutants were defective in the activities for either DNA binding or interacting with DNA bound Oct-1 or both, but were still capable of forming VIC in the presence of VCAF-1 indicated that neither the intrinsic DNA binding activity of Vmw65 nor the cooperativity with DNA-bound Oct-1 was a necessary prerequisite for the assembly of the full complex. These findings were consistent with our previous results using linker insertion mutants (Chapter 4).

When an increasing amount of MBP-Sal and mutants were added to the reactions containing a constant amount of GST-POU and VCAF-1, complexes corresponding to the Vmw65-DNA and Vmw65-POU-DNA were also visualized on the mobility shift gel. The titration experiments showed that when 0.5  $\mu\text{g}$  of MBP-Sal or a mutant fusion protein was incubated with 0.05  $\mu\text{g}$  of GST-



**Figure 5.6.6 Analysis of VIC formation with Vmw65 mutants**

The various MBP-Vmw65 mutants (0.5  $\mu$ g) were incubated with the (OCTA+)TAATGARAT DNA probe in the presence of GST-POU (0.05  $\mu$ g) and VCAF-1 (2  $\mu$ l). Lane A is the control containing only GST-POU. The contents of lanes B to K are indicated at the bottom of the figure.



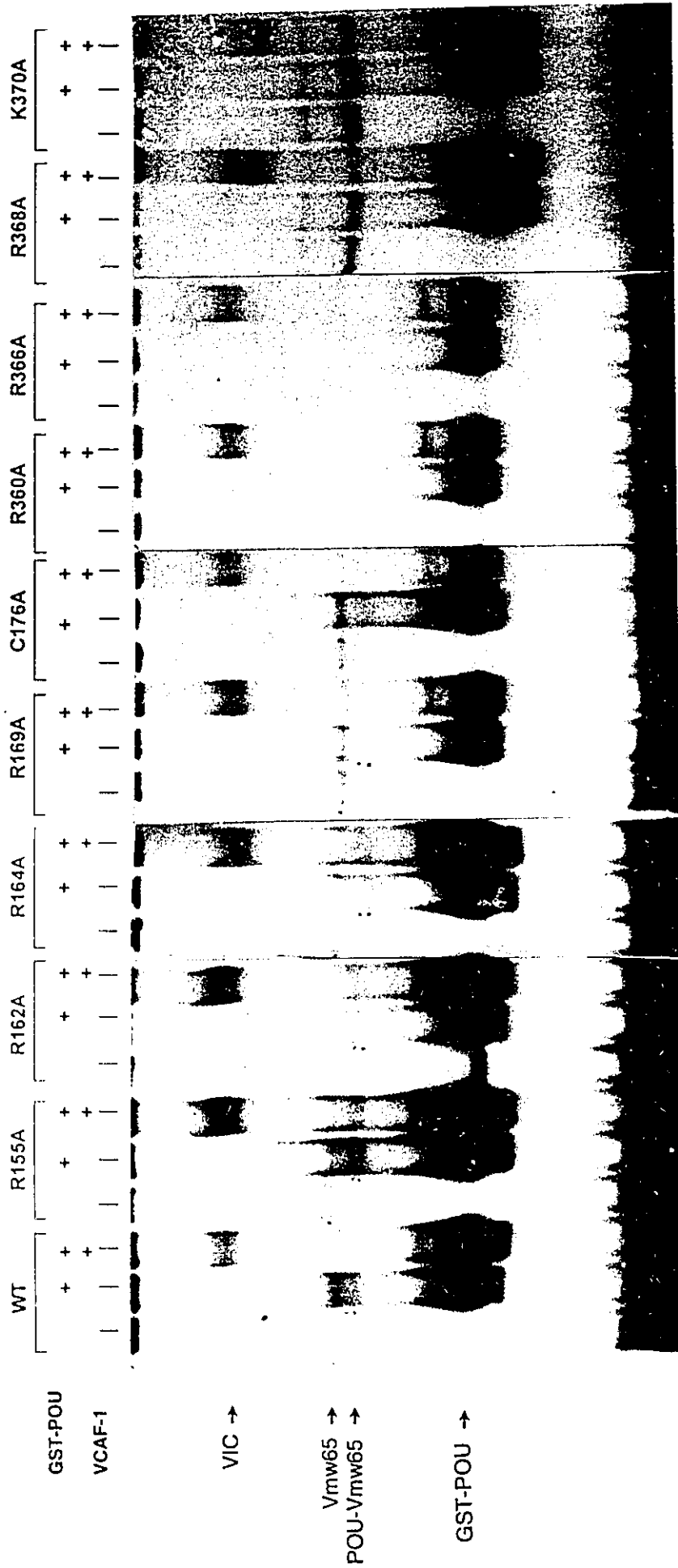
**Figure 5.6.7** Analysis of VIC formation with Vmw65 mutants using OCTA- DNA probe

The various MBP-Vmw65 mutants (6  $\mu$ g) were incubated with the (OCTA-)TAATGARAT DNA probe in the presence of GST-POU (0.05  $\mu$ g) and VCAF-1 (2  $\mu$ l). Lane A is the control containing only GST-POU. The contents of lanes B to K are indicated at the bottom of the figure.

POU and 2  $\mu$ l of VCAF-1, only the VIC complex was formed on the gel. However, when the amount of MBP-Sal and some mutants were increased to 8  $\mu$ g, the reactions containing same amount of POU and VCAF-1 were resolved in the VIC complex and two other faster migrating complexes corresponding to the Vmw65-DNA complex and the Vmw65-Oct-1 complex (Figure 5.6.8). The formation of Vmw65-DNA and/or Vmw65-Oct-1 complexes were consistent with the results obtained from the reactions, in which VCAF-1 was not added.

**5.7 In vivo induction of HSV IE gene promoters by transient transfection of Vmw65 expression vectors (the chloramphenicol acetyltransferase (CAT) assay)**

The assembly of the multi-component DNA binding complexes is necessary but not sufficient for the subsequent transactivation of IE genes *in vivo* by Vmw65 (Triezenberg et al., 1987; Greaves and O'Hare, 1989). Previous experiments by Werstuck and Capone (1989a,b) had further demonstrated that deletion mutants which reduced activation function of Vmw65 also partially impaired their ability to form the VIC complex with Hela cell extract. It is believed that the activation function of Vmw65 requires the assembly of the multi-component complex, in which the proper protein-protein interactions precisely place the activation domain of Vmw65 to the basal transcription machinery at the promoter. Therefore, if the individual protein-protein interactions involved in VIC



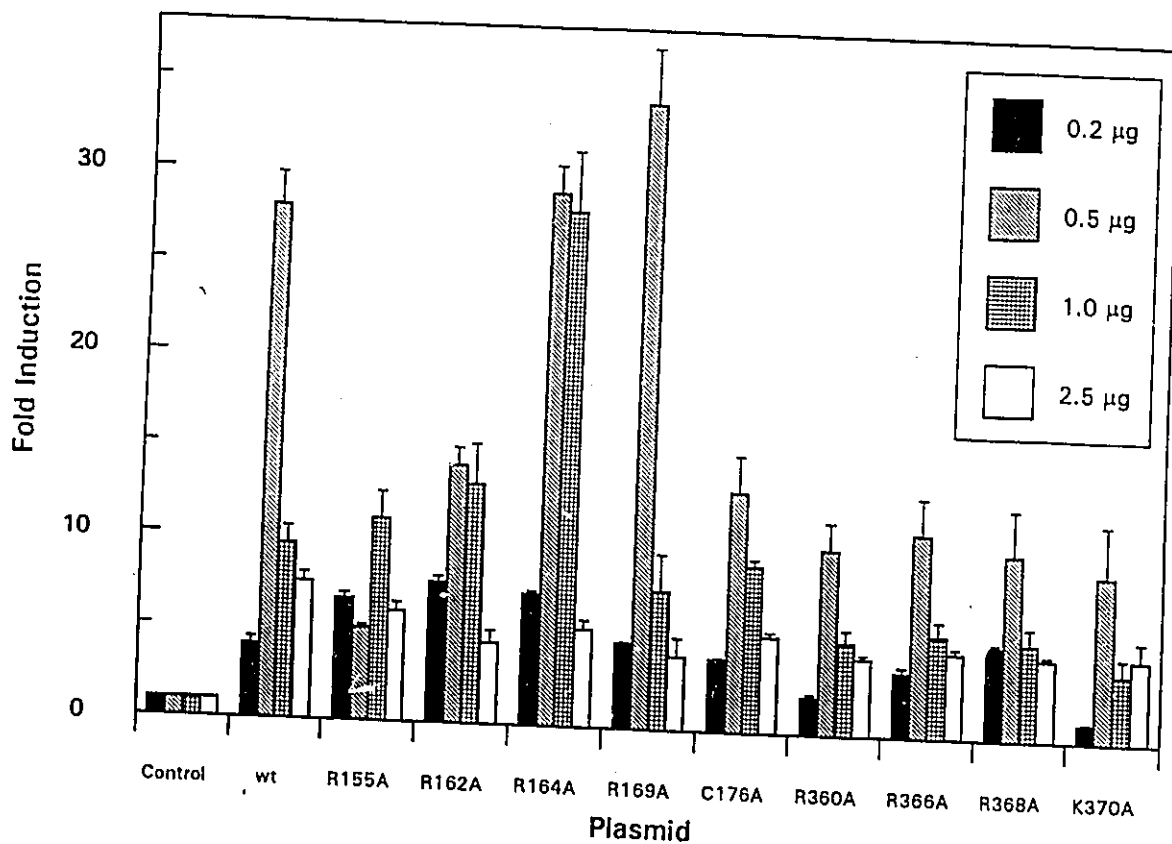
**Figure 5.6.8** Analysis of VIC formation with increased amount of MBP-Vmw65 mutants fusion proteins

The various MBP-Vmw65 mutants (6  $\mu$ g) were incubated with the (OCTA+)TAATGARAT DNA probe in the presence of GST-POU (0.05  $\mu$ g) and VCAF-1 (2  $\mu$ l), as indicated at the top of the figure. Vmw65 was used 10 fold excess as in figure 5.6.6 in order to visualize the Vmw65 and Vmw65-POU complexes, as indicated.



assembly were affected by the substitution of certain amino acids, the overall conformation of the full complex would be influenced and perhaps alter Vmw65 activation activity. To test the influence on activation by single amino acid substitutions, the various derivatives of Vmw65 were subcloned into an eukaryotic expression vector, pEVRF, in which the carboxyl terminal acidic activation domain was restored. The constructs were then transfected into cultured Vero cells with an ICP4 promoter controlled CAT reporter gene plasmid, p $\alpha$ 4CAT (constructed by Dr. Capone), to examine their activation abilities.

As shown in Figure 5.7.1, the wild type Vmw65 and all the mutant derivatives were able to induce the expression of CAT gene, and this was consistent with the observation that all mutants were capable of forming VIC in vitro. However, we have found the following interesting aspects: First, at 0.5 and 1.0  $\mu$ g level, the pEVRF DNA gave overall higher induction than at 0.2 and 2.5  $\mu$ g level. When 0.5  $\mu$ g of full-length wild type Vmw65 expression plasmid was co-transfected with 5  $\mu$ g of p $\alpha$ 4CAT, it stimulated approximately 30 fold of the expression of reporter gene. The reduction of activation activity caused by increasing amount of Vmw65 expression vector had been previously reported by Greaves and O'Hare (1989). The possible reason for the reduction is that the excess amount of expressed Vmw65 squelches the amount of a rate limiting cellular factor (possibly VCAF-1) that is required for VIC



**Figure 5.7.1** Substitution mutants are able to transactivate gene expression

Trans-activation of ICPO promoter controlled CAT gene was examined by transient transfection. 5  $\mu$ g of p $\alpha$ 4CAT was co-transfected with the indicated amount of eukaryotic expression plasmid, pEVRF inserted with the wild type and mutated Vmw65. The CAT activities were measured from 50  $\mu$ l of transfected cell extract by the organic solvent diffusion method. Shown here is the relative induction of CAT gene by wild type Vmw65 and mutant derivatives, as indicated.

assembly or activation. Second, there was large range of variations in relative activation activities among the different amino acid substitutions. Comparing their relative CAT activity at 0.5  $\mu$ g of plasmid, R164A and R169A had slightly higher induction than the wild type Vmw65, while the other mutants were only 30 to 40 percent effective as the wild type. However, at 2.5  $\mu$ g level, all the mutants had comparable activation activity as the wild type. The results indicated that the activation activity of the Vmw65 derivatives seemed to have no correlation with their ability to independently bind to DNA or VCAF-1. It was likely that the differences of stimulating activity of Vmw65 were caused by the relative stability of the proteins in vivo, since when higher amounts of plasmid was used, smaller differences were observed. And finally, with the exception of R155A, all mutants within region 1 had higher induction than those within region 2 when 0.5  $\mu$ g of plasmid was used. This result corresponded to previous observations that amino acid substitution in region 2 disrupted the cooperative interaction of Vmw65 with DNA-bound Oct-1.

## 5.8 SUMMARY

Table 5.8.1 summarizes the results of site-directed mutational analysis of Vmw65. Our study found that amino acids substitution mutants R162A, R360A and R366A were defective in intrinsic DNA binding to the TAATGARAT elements with or

**Table 5.8.1 Summary of mutational analysis of various requirements for Vmw65 with site-directed mutants**

Mutant	DNA Binding	Oct-1 Cooperativity	VCAF-1 Binding	VIC Assembly	Relative CAT Activity
W.T.	+	+	+	+	+++
Sal (5-411)	+	+	+	+	-
R155A	+	+	+	+	+++
R162A	-	+	+	+	+++
R164A	+	+	+	+	+++
R169A	+	+	-	+	+++
C176A	+	+	+	+	+++
R360A	-	-	+	+	++
R366A	-	-	-	+	++
R368A	+	-	-	+	++
K370A	+	-	-	+	++

without the overlapping octamer sequence. These results correspond to our previous findings that the independent DNA binding activity of Vmw65 involved in both regions 1 and 2. Stern and Herr (1991) also demonstrated that deletions in either region 1 or 2 interrupted DNA binding by Vmw65. In addition, they showed that a synthetic peptide containing residues 170-202 had non-specific DNA binding activity. Our findings that linker insertions at positions 178 and 241 destroyed the DNA binding activity of Vmw65 had also demonstrated the involvement of region 1 in DNA binding. However, the boundary of DNA binding domain of Vmw65 has not yet been defined. This study further demonstrated that both regions are involved in independent DNA binding by Vmw65.

Our study also showed that all amino acid substitutions at region 2 (e.g. R360A, R366A, R368A and K370A) eliminated the cooperativity with DNA-bound Oct-1, which is believed to confer selectivity to the POU domain and stabilize the interaction between Vmw65 and the DNA element (Herr and Cleary, 1995). This result is consistent with our previous findings that linker insertion at position 369 was able to bind to DNA but failed to cooperatively interact with POU, and C-terminal truncation mutant T-379 disrupted the cooperativity with DNA-bound Oct-1. It has been suggested that the interaction between Oct-1 and Vmw65 is mediated by region 2, which contains a homeodomain recognition subdomain (Stern and Herr, 1991; Hayes and O'Hare, 1993). Our results support these

findings.

The intrinsic DNA binding activity of Vmw65 was previously shown neither necessary nor sufficient for the cooperative interaction with DNA-bound Oct-1 (Chapter 4). Findings using site-directed mutagenesis support that conclusion since mutants R368A and K370A can bind to DNA directly but fail to interact with DNA bound Oct-1, while R162A is unable to bind to DNA but forms a complex with Oct-1 and DNA.

It has been previously demonstrated that the requirements for VIC formation can be uncoupled from the direct interaction with VCAF-1. Stern and Herr (1991) demonstrated that mutations in region 2 affected the ability of Vmw65 to interact with Oct-1 and form VIC but left DNA binding and VCAF-1 binding activities unchanged. Mutational studies using C-terminal truncations has demonstrated that the boundary for VIC formation is 388 (Greaves and O'Hare, 1990) whereas VCAF-1 can still interact with Vmw65 containing only 379 amino terminal residues (this thesis). It has been suggested that residues around lysine 370 within region 2 is involved in direct interaction with VCAF-1 since they are exposed on the surface of the protein and the synthetic peptide representing this region (amino acids 360-373) inhibited the formation of VIC (Hayes and O'Hare, 1993). However, there was no direct evidence to demonstrate this interaction. Our findings that mutations of R366A, R368A and

K370A abolished the direct interaction of Vmw65 with VCAF-1 directly showed that this subregion consists of an important interface for VCAF-1 binding. The findings that point mutant R169A (this thesis) and insertion mutant at amino acid 177 (Stern and Herr, 1991) impaired the VCAF-1 binding activity of Vmw65 also indicated the involvement of region 1 in the interaction.

Interestingly, all of the alanine substitution mutants were still able to form VIC in the presence of the Oct-1 and VCAF-1 *in vitro* and induce the expression of ICP4 promoter controlled CAT gene *in vivo*. These findings provide direct evidence that the interaction of Vmw65 with VCAF-1 in the absence of DNA and Oct-1 is not a necessary prerequisite for the assembly of a functionally active complex. This suggests that more than one pathway exists in the assembly of multi-component complex during the activation of IE genes by Vmw65. Presumably, with the presence of all viral and cellular components as well as DNA, the additional level of cooperative interactions among Vmw65, Oct-1 and VCAF-1 may occur to complement the deficiencies of individual interactions and facilitate the formation of the full complex. The protein-protein interactions among these factors may play important roles in differentiating these pathways. This flexibility could provide a mechanism by which Vmw65 is able to regulate the IE gene expression though adapting to different physiological conditions in host cell.

Upon infection of permissive host cells, it is possible that the concentration of VCAF-1 controls the process of lytic virus replication cycle. Under normal conditions, Vmw65 preferentially interacts with VCAF-1 and forms a binary complex, which effectively mediates the formation of full VIC complex with DNA-bound Oct-1 and subsequently activates the IE genes. Under certain cellular conditions, Vmw65 may have higher affinity with DNA-bound Oct-1 due to the conformational change of the POU domain induced by flanking regions around the octamer sequence (Walker *et al.*, 1994). The Oct-1-Vmw65-DNA complex may provide additional levels of cooperative interactions with VCAF-1, which stabilizes the binding of Oct-1-Vmw65 complex to DNA and facilitates assembly of the full VIC complex.

More recently, Wilson *et al.*, (1995a) demonstrated that the expression of VCAF-1 in proliferating cells is higher than in resting cells. They suggested that the requirement for Vmw65 to associate with VCAF-1 before activation IE genes might direct the proliferative status of infected cells, which influences whether the viral infection is followed by a lytic or latent pathway (Wilson *et al.*, 1995b). Our findings that prior interaction of Vmw65 with VCAF-1 is not necessarily required for complex formation and activation illustrates an additional layer of complexity in regulating viral genes by cellular factors. It is possible that in non-permissive cells, the expression and post-translational processing of VCAF-1 are



different from those in permissive cells. The second pathway of VIC assembly is predominant. Within those complexes, the AAD of Vmw65 does not have the proper conformation to activate downstream IE genes and results in a lysogenic pathway.

The flexibility involved in Oct-1 binding and complex assembly is also a mechanism for adapting the diversity of Vmw65 responsive elements in the remote region of HSV IE genes. The flexibility of POU domain in DNA binding enable Vmw65 to mediate the formation of multi-component complex on both (OCAT+) and (OCTA-) TAATGARAT elements in the presence of Oct-1 and VCAF-1 (Herr and Cleary, 1995 and references therein). However, how structural changes on Vmw65 would differentially affect the formation of the multi-component complex at these two types of TAATGARAT elements is unclear. To examine the influence of Vmw65 on VIC formation with (OCTA+) and (OCTA-) TAATGARAT elements, a radiolabelled (OCTA-) probe from ICP4 promoter was used to test the individual protein-protein and protein-DNA interactions, as well as the VIC formation with Vmw65 mutational derivatives. The results showed that the various Vmw65 mutants interacted with the (OCTA-)TAATGARAT element in a manner that was undistinguishable from the (OCTA+)TAATGARAT element in regards to intrinsic DNA binding activities, interactions with DNA bound Oct-1, and the formation of VIC in the presence of VCAF-1 and Oct-1. Therefore, the presence or absence of octamer sequence in the responsive element has no effects on the DNA

binding and complex formation properties of Vmw65 mutants. This finding indicated that these single amino acid substitutions were tolerated by the flexibility involved in directing the differential assembly of VIC complex.

The recent findings of transcriptional synergism involved in the activation of HSV IE genes have also provided evidence of additional levels of protein-protein interactions in the function of different types of TAATGARAT elements. Douville et al. (1995) reported that Vmw65 had higher efficiency of both activation and complex formation on (OCTA+) TAATGARAT element than that on (OCTA-). However, the presence of nearby CGGAAR motif significantly increased the inducibility and complex formation ability of Vmw65 on OCTA-TAATGARAT element. The synergism between VIC and GABP complexes may compensate the deficiencies of the Vmw65-induced complexes on OCTA- TAATGARAT element so that Vmw65 can achieve equivalent activation on both TAATGARAT responsive elements.

The elucidation of flexibility and its molecular basis involved in assembly of Vmw65 induced multi-component transcriptional complexes will continue to provide us better understanding to the selective and specific control of viral and cellular gene expression.

## CONCLUSIONS

The elucidation of the basis of gene regulation relies on our understanding of how transcription factors are selected and assembled onto *cis*-acting DNA elements and how distinct combinations and arrangements of these factors orchestrate differential gene expression. This thesis describes an effort to investigate the mechanisms by which eukaryotic gene expression is regulated through analysis of structure-function relationship of HSV transactivator Vmw65 in complex assembly and activation. By identifying a novel cellular factor required for the assembly of Vmw65 induced complex and examining distinct interactions among viral and cellular proteins during complex assembly, several properties of Vmw65 involved in complex assembly and transcriptional activation have been revealed.

The requirement of cellular factors for Vmw65 to activate viral genes provides a mechanism that allows viral gene expression to be regulated by multiple level of protein-protein interactions in diverse ways. This may allow HSV to adapt to different physiological conditions and efficiently utilize the cellular transcriptional machinery. The identification of the cellular factor VCAF-1 facilitates

further investigation of protein-protein interactions involved in selective assembly of multi-component complexes that control eukaryotic gene transcription.

The most significant functional characteristic of VCAF-1 is its high affinity with Vmw65 in the absence of DNA and other components of VIC. It is thus suggested that the formation of the VCAF-1-Vmw65 complex may be the initial step for assembling Vmw65 dependent complex. VCAF-1 might interact with the positively charged region of Vmw65 since it is negatively charged at normal physiological conditions. In addition, we first showed that VCAF-1 was possibly a protein factor due to its heat sensitivity and binding to protein affinity columns.

We established an *in vitro* system, by which the Vmw65 induced multi-component complex can be assembled in a stepwise manner using purified proteins. We subcloned and purified the POU domain of Oct-1, as well as expressed Vmw65 and mutants in different *E. coli* fusion protein systems.

We demonstrated that Vmw65 has weak intrinsic DNA binding activity when used in a high concentration. Although the putative domain of Vmw65 required for the intrinsic DNA binding activity of Vmw65 has not been defined, mutational analysis indicates that the carboxyl terminal boundary for the DNA binding activity of Vmw65 is located between 379 and 404. Using linker insertions and amino acid substitutions, we found that DNA binding activity of Vmw65 is distributed in regions

1 and 2 (141-250 and 335-390, respectively). The intrinsic DNA binding activity of Vmw65 apparently requires the GARAT portion of the TAATGARAT element although this element is not required for Oct-1 binding (Kristie and Sharp, 1990).

Vmw65 is able to cooperatively interact with DNA-bound Oct-1. We identified the region containing amino acids 379-404 as essential for the cooperativity with Oct-1. This is consistent with reports by others that the region required for Vmw65 to cooperatively interact with DNA-bound Oct-1 located to amino acids 378-389 (Stern and Herr, 1991) or 375-385 (Hayes and O'Hare, 1993). Our site-directed mutagenesis study further demonstrated that all mutants at region 2 eliminated the cooperativity with DNA-bound Oct-1, indicating the involvement of region 2 in cooperative DNA binding.

The intrinsic DNA binding activity or cooperative interaction with DNA-bound Oct-1 seem not to be required for Vmw65 to mediate complex formation since mutants defective in either or both properties were still capable of generating VIC when all necessary components co-existed.

The independent interaction between Vmw65 and VCAF-1 was investigated using micro-affinity assays. We found that both regions 1 and 2 of Vmw65 are involved in the independent VCAF-1 binding activity. Using deletion mutants, we first found that the requirements for VIC formation overlapped but were distinct from that required for independent interaction with VCAF-1. This indicated that the requirement for VCAF-1

binding can be uncoupled from complex formation. Using site-directed mutations, we further demonstrated that the requirement for prior interaction of Vmw65 with VCAF-1 was not necessary for VIC assembly and transcriptional activation.

Our findings suggested that more than one pathway exists in cooperative assembly of multi-component complexes during the activation of IE genes by Vmw65. The protein-protein interactions among these factors may play important roles in differentiating these pathways. Presumably, with the presence of all viral and cellular components as well as DNA, additional levels of cooperative interactions among Vmw65, Oct-1 and VCAF-1 may occur to compensate the deficiencies of individual interactions and facilitate the formation of the full complex.

Upon infection of the host cell, it is possible that the differential expression and processing of VCAF-1 controls the progress of lytic virus replication cycle. In permissive cells, Vmw65 preferentially interacts with VCAF-1 and forms a binary complex, which effectively mediates the formation of full VIC complex with DNA-bound Oct-1 and subsequently activates the IE genes to initiate lytic cycle. Whereas in non-permissive cells, Vmw65 has higher affinity with DNA-bound Oct-1. The binary Vmw65-Oct-1 complex then facilitates the binding of VCAF-1, which further stabilizes the tertiary complex at the DNA element. However, the different arrangement of complexes in these complexes may confer different

activation function to Vmw65.

Finally, the flexibility of POU domain in recognition of different octamer sequences may directly influence its participation in the multi-complex assembly with Vmw65 and VCAF-1. It provides a mechanism for Vmw65 to adapt to the diversity in responsive elements of HSV IE genes. The flexibility of the POU domain in DNA binding enables Vmw65 to mediate the formation of multi-component complex on either OCTA+ or OCTA- promoter element in the presence of Oct-1 and VCAF-1 (Herr and Cleary, 1995). Our findings also showed that various Vmw65 mutants interacted with the (OCTA-) TAATGARAT element in the same manner as with the (OCTA+) TAATGARAT element in terms of DNA binding, cooperativity with DNA-bound Oct-1, and VIC formation in the presence of VCAF-1 and Oct-1. The individual deficiencies caused by substitution of single amino acid residue can be tolerated by the flexibility involved in directing the differential assembly of VIC complex.

In addition, we demonstrated that VHS inhibits the ability of Vmw65 to participate in VIC assembly on the TAATGARAT elements (see appendix). This implies that VHS may directly affect the role of Vmw65 in viral gene expression.

Future investigations of transcriptional activation by Vmw65 will help elucidate diversity of molecular basis involved in assembly of Vmw65 induced multi-component transcriptional complexes that selectively and specifically control viral and cellular gene expression.

## Appendix

### THE INTERACTION BETWEEN VMW65 AND HOST CELL SHUT-OFF FACTOR (VHS) AND THE ROLE OF VHS IN REGULATING IE GENE EXPRESSION

Besides Vmw65, which activates the expression of IE genes of HSV upon viral infection, HSV contains one or more viral structural proteins that participate in the early events of viral gene expression by strongly suppressing cellular gene expression and inducing the accelerated turnover of cellular and viral mRNA (Nishioka and Silverstein, 1977; Fenwick and McMenamin, 1984; Schek and Bachenheimer, 1985). One of these factors has been identified as a late gene (UL41) product and is packaged into the virion tegument. Viral host shut off factor (VHS) is a 489 amino acid phosphoprotein with a molecular mass of 58 kDa (Fenwick and Everett, 1990; Smibert et al., 1992). Previous experiments carried out by Smibert et al. (1994) using polyclonal antiserum against VHS showed that Vmw65 was co-immunoprecipitated with VHS from the HSV infected Hela cells. Using affinity chromatography with protein A fused VHS, they further demonstrated that VHS had direct binding activity to Vmw65 and the carboxyl terminal activation domain of Vmw65 was not required for the interaction. Mutagenesis studies revealed that VHS residues 238 to 344 were sufficient for the interaction with Vmw65 and the truncation mutant T-335



of Vmw65 was still able to bind to VHS. However, the interaction between VHS and Vmw65 had not been observed in the presence of the Vmw65 responsive DNA element. In order to investigate whether the independent interaction between Vmw65 and VHS affected the assembly of the multi-component activation complex, the interaction of Vmw65 with VHS in the presence of Oct-1, VCAF-1 and the TAATGARAT element was examined with *in vitro* gel shift assay.

#### 6.1 Characterization of the interaction between Vmw65 and VHS *in vitro* using a mobility shift analysis

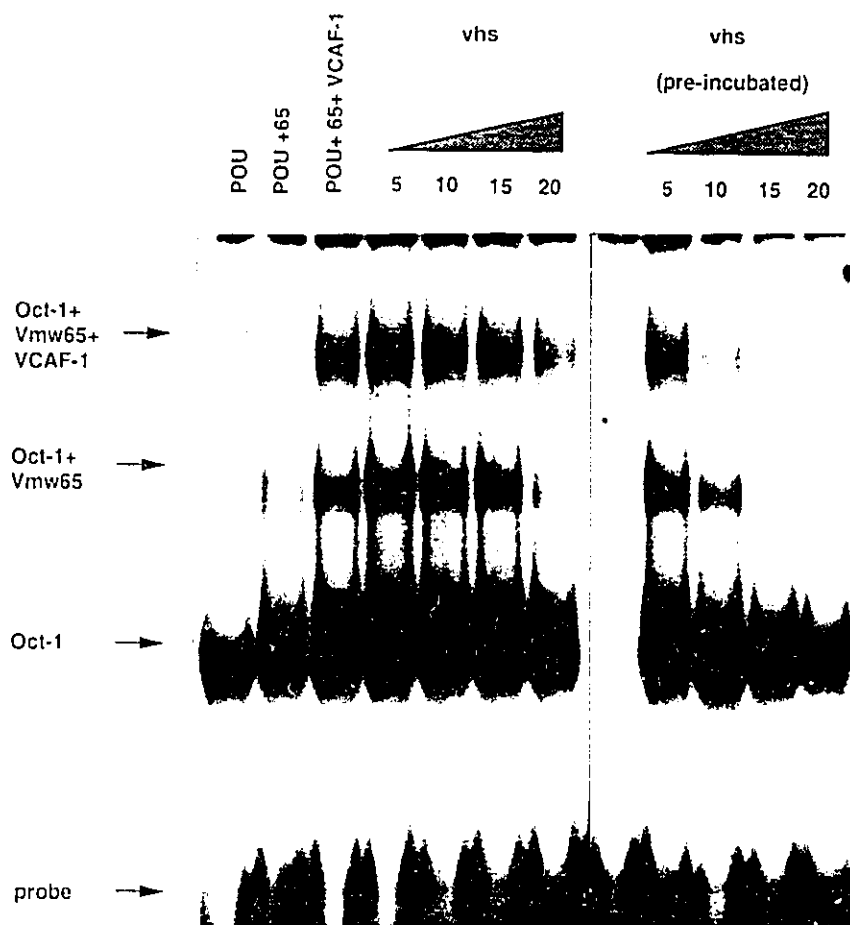
Protein-DNA mobility shift assays were carried out with two separate sets of incubations. Standard reactions contained 5 mM HEPES, pH 7.9, 0.1 mM DTT, 0.5 mM EDTA, 25 mM KCl, 4  $\mu$ g of competitor DNA (a 1:2 mixture of salmon sperm DNA-poly dI-dC) with the final volume of 15  $\mu$ l. In the first set of incubations, the reaction mixture also contained 1  $\mu$ g of protein A fused POU domain of Oct-1 (PA-POU), 0.5  $\mu$ g of PA-65, affinity purified VCAF-1 and titrating amount of PA-VHS from 5  $\mu$ g to 20  $\mu$ g. The protein concentration in each reaction was normalized with BSA. The second set contained the same mixture as the first, with the exception that PA-POU and purified VCAF-1 were added after PA-65 and VHS were preincubated at 30°C for 30 minutes. The reaction mixtures of both sets were preincubated for 5 minutes at room temperature prior to the addition of 0.15 pmol of <sup>32</sup>P-labelled TAATGARAT

probe. The reactions were then incubated at 30°C for 20 minutes and applied on mobility shift gels.

## 6.2 High concentration of VHS blocks the ability of Vmw65 to mediate the assembly of multi-component DNA binding complex

The mobility shift assay resolved three protein-DNA complexes: a rapid migrating complex containing only PA-POU, an intermediate complex containing both PA-POU and PA-65, and the fully assembled complex containing PA-POU, PA-65 and VCAF-1 (Figure 6.1.1). It was found that the excess amount of PA-VHS had only a minor effect on the complex formation when it was added at the same time with other components. However, when PA-VHS was pre-incubated with Vmw65 prior to the addition of PA-POU, VCAF-1 and DNA probe, an excess amount of PA-VHS greatly inhibited the formation of complexes containing Vmw65. Moreover, PA-VHS had no effect on complex containing only PA-POU and DNA probe. This result suggested that Vmw65 was blocked from participating in the assembly of VIC on TAATGARAT element when it was bound with VHS. It implied that VHS could be directly involved in the interactions among Vmw65, Oct-1 and VCAF-1 during complex assembly. However, the affinity between Vmw65 and VHS seems weaker than that between Vmw65 and host cell factors since the inhibition of the VIC formation was only observed when VHS was pre-incubated with Vmw65 with excess amounts. Under physiological conditions, the amount of

Vmw65 is much more abundant than VHS in both the virion and infected cells, it is more likely that Vmw65 regulates the function of VHS rather than VHS regulates the expression of immediate early genes of HSV by blocking Vmw65 from entering transactivation complex assembly.



**Figure 6.1.1 The VHS protein blocks VIC assembly.** The DNA probe was incubated with PA-POU, PA-65, and VCAF-1. In the first set of reactions, the indicated quantity of PA-VHS ( $\mu\text{g}$ ) was added at the same time as the other components. In the second set of reactions, Vmw65 and VHS were preincubated for 30 min prior to the addition of PA-POU and VCAF-1. DNA-protein complexes were resolved on a 3.5% native gel.

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