

**PROPERTIES AND FUNCTION OF THE HSV TRANSACTIVATOR VP16
EXPRESSED IN YEAST *SACCHAROMYCES CEREVISIAE***

**PROPERTIES AND FUNCTION OF THE HSV TRANSACTIVATOR VP16
EXPRESSED IN YEAST *SACCHAROMYCES CEREVISIAE***

By

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ABSTRACT

Herpes simplex virus protein VP16 activates immediate-early (IE) viral gene expression upon infection. VP16-mediated transactivation depends on formation of a multiprotein complex with cellular factors on a *cis*-acting TAATGARAT sequence present in the IE promoters. The potent acidic activation domain, contained within the carboxyl terminus of VP16, is dispensable for the complex formation. The amino terminal part of VP16, which is inert in transactivation in mammalian cells, is sufficient for selective interactions with cellular factors, one of which has been identified as the ubiquitous transcription factor Oct-1.

The yeast two-hybrid system was utilized to isolate the cellular factor(s) necessary in addition to Oct-1 for VP16 induced complex formation. This system, designed to directly clone proteins interacting with a given protein of interest, employs the yeast transcriptional activator GAL4. An interaction between VP16 and the cellular factor(s), fused to GAL4 DNA binding and activation domain, respectively, reconstitutes a hybrid transactivator that stimulates expression of a reporter lacZ gene in yeast. Thus, β -galactosidase activity serves as a positive signal for protein-protein interaction.

As a prelude of using this method for isolation of VP16-interacting cellular proteins, the system was tested with HSV-1 protein vhs, known to bind to VP16 *in vitro*.

The obtained data demonstrated an interaction between VP16 and vhs in the two-hybrid system and deletion analysis revealed that VP16 sequence contained within the first 369 amino acids is required for binding to vhs. Thus, VP16 residues necessary for interaction with vhs *in vivo* coincide with these identified previously for VP16-vhs complex formation *in vitro*.

VP16 fused to the GAL4 DNA binding domain activated expression of the reporter lacZ gene in yeast, despite the absence of its acidic activation domain. Deletion analysis showed that the amino terminal 369 residues of VP16 were sufficient for transactivation in yeast. Similar GAL4-VP16 derivatives were inactive in mammalian cells as measured by transient transfection assays. Thus, unlike in yeast, VP16 lacking the acidic activation domain is deficient in transactivation in mammalian cells even if it is directly bound to a promoter.

VP16 sequences required for complex formation with vhs overlaps with those implicated in interaction with the mammalian factors, indicating that this region is involved in protein-protein interactions with both cellular and viral factors. Consistent with this, VP16 interaction with a yeast factor supplying an activation domain *in trans* would explain VP16-dependent transactivation in the absence of its acidic activation domain. Alternatively, a yeast specific activation domain might be present in the amino terminal part of VP16.

DEDICATIONS

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ABBREVIATIONS

AAD	acidic activation domain
AD	activation domain
ADH	alcohol dehydrogenase
amp	ampicillin
APS	ammonium persulphate
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
CAT	chloramphenicol acetyl transferase
CEN	centromere sequence
Ci	Curie
CIP	calf intestine phosphatase
Da	Dalton
DBD	DNA binding domain
DME	Dulbecco modified essential medium
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
DOC	deoxycholate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid
h	hour(s)
HEPES	n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIS	histidine
HRP	horseradish peroxidase

HSV-1	herpes simplex virus type 1
IE	immediate-early
LEU	leucine
LiAc	lithium acetate
μ	micro
m	milli
M	molar
MCS	multiple cloning site
min	minute(s)
Mw	molecular weight
NMR	nuclear magnetic resonance
NP-40	Nonidet P-40
NTPs	ribonucleotides
ONPG	o-nitrophenyl- β -D-galactoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
pol II	RNA polymerase II
RNA	ribonucleic acid
RT	room temperature
rpm	revolutions per minute
Sc	synthetic complete medium
SDS	sodium dodecyl sulphate
TAF	TBP associated factor

TBP	TATA-box binding protein
TBST	Tris-buffered saline - Tween
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TEN	Tris-EDTA-NaCl
TF	transcription factor
Tris	Tris(hydroxymethyl) aminoethane
TRP	tryptophan
ts	temperature sensitive
UV	ultra-violet
VCAF-1	VP16 complex assembly factor
vhs	virion-induced host shutoff
VIC	VP16 induced complex
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
YEPD	yeast extract/peptone/dextrose medium

1. INTRODUCTION

1.1. Transcription by RNA polymerase II

Basal transcription by RNA polymerase II (Pol II) requires the assembly of a set of general transcription factors on the core promoter element. The general transcription factors necessary for preinitiation complex formation involve the TATA-box binding protein (TBP) with TBP-associated factors (TAFs), TFIIA, TFIIB, TFIIE, TFIIF, TFIIIG, TFIIH, TFII-I and TFIIJ (reviewed in Conaway and Conaway, 1993; Zawel and Reinberg, 1993). Both protein-protein and protein-DNA interactions mediate formation of the transcription preinitiation complex. The general transcription factors bind to the core promoter element in an ordered pathway (reviewed in Zawel and Reinberg, 1992; Drapkin *et al.*, 1993; Corden, 1993). The first step is binding of TFIID, which consists of TBP and different TAFs (Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; reviewed in Gill and Tjian, 1992), to the TATA or Initiator element, forming the so called initial complex (Conaway and Conaway, 1993). TFIID binding is stabilized consequently by TFIIA. This is followed by binding of TFIIB, RNA PolII/TFIIF, TFIIE, TFIIH and TFIIJ to form complete but inactive (also called closed) preinitiation complex. Activated (or open) preinitiation complex is formed upon addition of ATP and transcription starts in the presence of the NTPs (Wang *et al.*, 1992).

The level of transcription is regulated by transactivators bound to specific DNA sequences usually located upstream of the initiation site (reviewed in Ptashne, 1988). These transactivators are often modular in structure, containing separable and functionally independent DNA binding and activation domains, respectively (reviewed in Mitchell and Tjian, 1989). The level of gene expression is dependent both on the particular combination of the *cis*-acting DNA elements and on specific interactions between transactivators and the general factors. These interactions are thought to facilitate the assembly of the preinitiation complex, to stabilize the complex, or to increase the number of active complexes (Ptashne, 1988).

Additional factors, are often required to mediate the interaction between the transactivators and the general factors (Berger *et al.* 1990; Kelleher *et al.* 1990; Pugh and Tjian, 1990). These factors, variously termed adaptors, co-activators or mediators, are necessary for activated but not basal transcription. As the adaptors do not have specific DNA binding activity, they are thought to serve either as a bridging molecule between the activator and the general factor or to stabilize a direct interaction between the transactivator and general factor (reviewed in Gill and Tjian, 1992).

1.2. Viral proteins as a model system for elucidating the mechanism of transcriptional control

The regulation of transcription in eukaryotic cells is achieved through the coordinate action of transactivators and general transcription factors assembled into

multicomponent complexes on the promoter elements through protein-DNA and protein-protein interactions. Elucidating the mechanism of complex formation and the nature of the selective interactions between the different regulatory proteins is necessary for understanding the basis of transcriptional control. Viruses that infect eukaryotic cells often possess proteins able to interfere with and re-direct the cellular transcriptional machinery for the expression of the viral genes. The mechanism of action of these viral transactivators is similar to that utilized by the cellular transcription factors (reviewed in Martin and Green, 1992). Transactivation of viral genes induced by herpes simplex virus (HSV) protein VP16 is dependent upon specific interactions with cellular factors resulting in formation of a multicomponent complex on the viral promoter sequence. Thus, VP16 provides a favourable model system for study the role of selective protein-DNA and protein-protein interactions in the combinatorial control of gene expression (Goding and O'Hare, 1989). Elucidating the mechanism of this process is important for better understanding not only the nature of HSV-1 caused infection, but also for illuminating the complex transcription regulatory circuits normally operating in eukaryotic cells.

1.3. Herpes simplex virus transactivator VP16

VP16 (also known as Vmw65, α TIF, ICP25) is HSV-1 phosphoprotein, located in the tegument structure of the virion (an amorphous protein layer between the nucleocapsid and the envelope). It is one of the most abundant HSV-1 proteins, found at approximately 500 to 1000 molecules per particle (Heine *et al.*, 1974). VP16 is a

major structural component of the virion and as such has an essential role for virus assembly (Ace *et al.*, 1988); virus deleted for the VP16 open reading frame (ORF) is unable to propagate in host cells (Weinheimer *et al.*, 1992).

Apart from its structural role, VP16 is a potent transactivator involved in activation of the immediate early (IE, α) viral gene expression upon infection. Evidence for stimulation of the IE gene transcription by a virion component came from studies demonstrating induction of IE gene expression in the absence of *de novo* protein synthesis (Post *et al.*, 1981), as well as upon infection with either UV light-inactivated virus or with a temperature sensitive (ts) mutant defective in the release of viral DNA from the capsid (Batterson and Roizman, 1983). A region of HSV-1 genome whose product stimulated specifically IE promoters was isolated later on (Campbell *et al.*, 1984) and consequent DNA sequencing identified the VP16 gene as responsible for this transactivation (Dalrymple *et al.*, 1985; Pellet *et al.*, 1985). The deduced VP16 sequence encodes a 490 amino acid polypeptide with predicted molecular weight (Mw) 54342 Da. The protein is highly negatively charged with the acidic residues concentrated in the carboxy-terminal 80 amino acids, while the basic residues are prevalent near the amino-terminal 160-210 amino acids (Dalrymple *et al.*, 1985).

VP16 transactivation of IE gene expression is thought to facilitate the establishment of lytic infection. Although viable, a HSV-1 mutant defective in VP16 transactivation is severely impaired in its ability to form plaques at low multiplicity of infection (Ace *et al.*, 1989), which likely corresponds to the physiological conditions in

the infected host.

The VP16 mediated transactivation is dependent on a *cis*-acting regulatory element TAATGARAT (where R is a purine) present in at least one copy in the promoter region of each of the IE genes (Mackem and Roizman, 1982; Kristie and Roizman, 1984; Preston *et al.*, 1984; Gaffney *et al.*, 1985). Although VP16 is able to recognize (O'Hare *et al.*, 1988) and bind specifically to the GARAT part of the consensus sequence (Kristie and Sharp, 1990; Stern and Herr, 1991), this interaction is of low affinity. Stable and specific binding of VP16 to its target sequence is achieved through cooperative interaction with additional cellular proteins, forming a multicomponent complex, designated VIC (for VP16 induced complex), IEC, or TRF.C (McKnight *et al.*, 1987; Preston *et al.*, 1988; O'Hare and Goding, 1988). Formation of this complex is a prerequisite for the IE gene induction, as VP16 mutants defective in VIC assembly are impaired in transactivation as well (Ace *et al.*, 1988).

One of the components of this multiprotein complex was identified as the ubiquitous octamer binding protein Oct-1 (also known as OTF-1, NF-III, OB100) (Gerster and Roeder, 1988; O'Hare *et al.*, 1988; Stern *et al.*, 1989; Kristie *et al.*, 1989), a member of POU domain transcription factors family. The TAATGARAT element present in the viral IE promoters often overlaps with the consensus octamer motif ATGCAAAT. Oct-1 POU domain alone binds with high affinity to VP16 target sequence (Kristie *et al.*, 1989; Stern *et al.*, 1989) and Oct-1 homeodomain is sufficient for promoting VIC assembly on TAATGARAT element (Stern *et al.*, 1989; Kristie and

Sharp, 1990). The recruitment of Oct-1 into VIC is highly specific as the B-cell specific transcriptional factor Oct-2, which has a high degree of homology with Oct-1 POU domain and binds to the same octamer element, is not able to substitute for Oct-1 in VP16 induced complex (Gerster and Roeder, 1988; Stern *et al.*, 1989). A single glutamic acid for alanine difference in helix 1 of Oct-1 homeodomain is the major determinant responsible for the selective association of VP16 with Oct-1 but not with Oct-2 (Lai *et al.*, 1992; Pomerantz *et al.*, 1992), illustrating the high specificity of protein-protein interactions among the regulatory factors.

Binding of Oct-1 to TAATGARAT element increases the affinity of VP16 binding to DNA (Kristie and Sharp, 1990; Stern and Herr, 1991), but formation of a stable complex requires additional cellular factor(s) (Gerster and Roeder, 1988; Kristie *et al.*, 1989; Kristie and Sharp, 1990; Xiao and Capone, 1990; Katan *et al.*, 1990), variously termed VCAF-1 (for VP16 complex assembly factor), C1, HCF or CFF. Unlike Oct-1, VP16 interacts directly with VCAF-1 in the absence of target DNA (Xiao and Capone, 1990; Kristie and Sharp, 1990). As VCAF-1 does not have sequence specific DNA binding activity (Xiao and Capone, 1990; Kristie and Sharp, 1990; Katan *et al.*, 1990), its interaction with VP16 might form an intermediate complex with higher affinity for DNA bound Oct-1. Extensive chromatographic purification aimed at the isolation of VCAF-1 revealed a set of six closely related polypeptides ranging from 100- to 150 kDa and one larger protein of 300 kDa (Kristie and Sharp, 1993; Wilson *et al.*, 1993). The individual polypeptides seems to be completely inactive for VIC formation (Kristie and

Sharp, 1993). The gene encoding for HCF (VCAF-1) was recently isolated and shown to encode for a large ORF of 2035 codons corresponding to the 300 kDa protein as well as to the smaller polypeptides, with no obvious sequence similarity to other known proteins (Wilson *et al.*, 1993). It has been suggested that the 300 kDa protein is the primary translation product which subsequently is proteolytically cleaved into the smaller polypeptides.

1.4. Domain structure of HSV-1 protein VP16

As with many transcriptional activators, VP16 has a modular structure comprising two functionally independent and separable domains, one of which contributes to the specific association on TAATGARAT sequence, while the other is involved in transactivation.

A potent acidic activation domain (AAD) which is essential for transactivation but dispensable for complex formation, is contained within the carboxy-terminal 78 amino acids (Triezenberg *et al.*, 1988; Greaves and O'Hare, 1989). Fusion of these 78 amino acids to a heterologous DNA binding domain results in construction of a strong transactivator stimulating transcription from responsive promoter both in yeast and mammalian cells (Sadowski *et al.*, 1988; Cousens *et al.*, 1989). The net negative charge of VP16 activation domain is important but not the only determinant required for transactivation (Cress and Triezenberg, 1991). Formation of an amphipathic α -helix (according to the secondary structure predictions) was thought to contribute to the

activation function (Cousens *et al.*, 1989), however, no correlation between transcriptional activation and the predicted α -helicity or amphipathy was found through mutational analysis of VP16 (Cress and Triezenberg, 1991). Consistent with this, circular dichroism spectroscopy (Donaldson and Capone, 1992) and nucleic magnetic resonance analysis (O'Hare and Williams, 1992), did not reveal a presence of α -helical conformation of the acidic activation domain under physiological conditions.

The cellular targets of VP16 acidic activation domain have been studied extensively. Evidence for specific interactions with the general transcription factors TFIIB (Lin and Green, 1991; Lin *et al.*, 1991; Roberts *et al.*, 1993; Walker *et al.*, 1993), TFIID (Stringer *et al.*, 1990; Ingles *et al.*, 1991) and TFIIF (Reinberg and Zawel, 1993), transcriptional adaptors (Berger *et al.*, 1990; Kelleher *et al.*, 1990; White *et al.*, 1991; Berger *et al.*, 1992), and replication factor A (He *et al.*, 1993; Li and Botchan, 1993) have been reported. It is possible that multiple interactions with several factors are required for VP16 mediated transactivation (Hahn, 1993; Walker *et al.*, 1993).

The amino-terminal part of VP16 is involved in protein-protein and protein-DNA interactions with Oct-1 and VCAF-1 resulting in VIC assembly on TAATGARAT sequence. The acidic activation domain is completely dispensable for VIC formation and does not affect the assembly or stability of the complex (Greaves and O'Hare, 1989; Werstuck and Capone, 1989b; Greaves and O'Hare, 1990; Xiao and Capone 1990). On the contrary, VIC assembly is necessary but not sufficient for stimulation from IE

promoters, as VP16 devoid of its activation domain, promotes complex formation but is completely deficient in transactivation (Ace *et al.*, 1988). Thus, the assembly of this complex on TAATGARAT sequence is important in order to bring the potent VP16 activation domain in vicinity to the transcriptional machinery and stimulate transcription of the IE genes. The requirements for VIC assembly have been mapped to the first 388 amino acids of VP16 (Greaves and O'Hare, 1990). A region encompassing amino acids 360-391 has been implicated in binding to Oct-1 POU domain and VCAF-1 (Stern and Herr, 1991; Hayes and O'Hare, 1993), while a small domain located between residues 170-202, which constitutes of the most basic region of the protein, is thought to be important for DNA binding activity of VP16 (Stern and Herr, 1991).

Thus, multiple distinct regions of VP16 are involved in specific protein-protein and protein-DNA interactions, resulting in VIC formation on TAATGARAT element and consequent transactivation of the IE gene expression.

1.5. The two-hybrid system for identification of the cellular factor(s) interacting with VP16 lacking the acidic activation domain

The transactivation function of VP16 depends on its interaction with the cellular factors to form the VP16 induced complex on TAATGARAT element.

At the time this work began, the ubiquitous Oct-1 protein was identified as one of the components of this multiprotein complex. The necessity of the additional cellular factor(s), which is referred here as VCAF-1, had been shown by several laboratories

(Gerster and Roeder, 1988; Kristie *et al.*, 1989; Kristie and Sharp, 1990; Xiao and Capone, 1990; Katan *et al.*, 1990), but the protein and the corresponding gene were not known. Thus, the object of my project, initially, was to attempt to isolate the cellular factor(s) necessary in addition to Oct-1 for VP16 induced complex formation. As the presence of this factor(s) in cells is extremely low, its purification and isolation using conventional chromatographic methods (including affinity chromatography) is difficult to accomplish. Thus, the yeast two-hybrid system was employed as an alternative and more feasible approach for directly isolating the DNA coding for VP16-interacting cellular protein(s).

The two-hybrid system is designed for cloning DNA encoding for proteins interacting with a given protein of interest (Fields and Song, 1989; Chien *et al.*, 1991). The detection of protein-protein interaction *in vivo* is based on reconstitution of activity of yeast transcriptional activator GAL4 (required for the expression of genes encoding enzymes for galactose metabolism). The system employs the fact that GAL4 consists of two separable and functionally independent domains: a DNA binding domain (DBD) (amino acids 1-147) that directs the protein to GAL4 binding site, but is deficient in transactivation, and an activation domain (AD) (residues 768-881) which does not have DNA binding ability on its own. The protein of interest (e.g. VP16 lacking the AAD) is fused to GAL4 DBD, while protein sequences encoded from a library are fused to GAL4 AD. Both hybrid proteins are expressed in yeast strain deleted for GAL4 and GAL80 (a negative regulator of GAL4) and harbouring a resident lacZ reporter gene

under control of GAL4-responsive promoter. GAL4-DBD fusion protein binds to its cognate sequence but does not induce lacZ gene expression, as it lacks an activation domain. Transactivation of the reporter gene does not occur with GAL4-AD hybrid, either, as it is not able to bind to GAL4 binding sites. However, an interaction between the protein under study (VP16) with a protein from the library brings GAL4 activation domain to GAL4 DNA binding domain and restores the activation function, resulting in expression of the lacZ gene. Thus, detection of galactosidase activity serves as a positive signal for identification the proteins interacting with VP16.

As a prelude of using the yeast two-hybrid system for identifying cellular proteins interacting with VP16 lacking the AAD, we tested the system with another HSV-1 protein, vhs, known to form a complex with VP16 *in vitro* (Smibert *et al.*, 1993). Thus, the first section of the presented results describes the study of interaction between VP16 and vhs *in vivo* using the yeast two-hybrid system.

VP16 fused to GAL4-DNA binding domain was alone sufficient to activate transcription of the reporter lacZ gene in yeast, despite the absence of its acidic activation domain. The second section presents the results of the study of transactivation function of VP16 lacking the AAD in yeast.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The chemicals and the reagents utilized in this study as well as the companies from which they were purchased are listed below:

amino acids	Sigma Chemical Company
ampicillin	Sigma Chemical Company
bacto-agar	Difco Laboratories
bacto-peptone	Difco Laboratories
BioRad protein assay	BioRad Laboratories
5-Bromo-4-chloro-3-indolyl- β -D-galactoside	GIBCO BRL
ECL Western blotting detection reagents	Amersham
deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)	Pharmacia
dimethyl sulfoxide	BDH Chemicals
dithiothreitol	Sigma Chemical Company

molecular weight standards:

(i) 1kb DNA ladder

GIBCO BRL

(ii) Prestained SDS-PAGE standards

(Low range)

BioRad Laboratories

nitrocellulose membrane

Schleicher and Schuell Inc.

o-nitrophenyl β -D-galacto-pyranoside

Sigma Chemical Company

Nonidet P-40 (NP-40)

United States Biochemical Corp.

NuSieve GTG agarose

FMC BioProducts

Silica gel 150A plates

Whatman

Triton X-100

BioRad Laboratories

Tween 20

BioRad Laboratories

yeast extract

Difco Laboratories

yeast nitrogen base (without amino acids)

Difco Laboratories

2.1.2. Radiochemicals

chloramphenicol, [dichloroacetyl-

1,2- ^{14}C]; (58.2 mCi mmol $^{-1}$)

New England Nuclear Corp.

$[\alpha\text{-}^{32}\text{P}]$ -dATP (3000 Ci mmol $^{-1}$)

ICN Radiochemicals, through the
laboratory of Dr.C.Harley, Dept. of
Biochemistry, McMaster University

2.1.3. Enzymes

All enzymes were used according to the recommendations of the manufacturers.

Calf intestine phosphatase	Pharmacia
DNA ligase (T4)	New England Biolabs (NEB)
DNA polymerase I (Klenow)	NEB
DNA polymerase, modified T7 (Sequenase, Version 2.0)	United States Biochemical Corp.
Mung bean nuclease	Pharmacia
restriction endonucleases	GIBCO BRL; NEB
RNase A	Pharmacia

2.1.4. Antisera

GAL4 polyclonal antibody was a gift from Dr. Ivan Sadowski, Dept. of Biochemistry, University of British Columbia.

Anti-rabbit Ig, horseradish peroxidase linked whole antibody (from donkey) was purchased from Amersham.

2.1.5. Yeast fusion vectors

Plasmids pPC97 and pPC86 (Chevray and Nathans, 1992), were a generous gift from Dr. Pierre Chevray, Dept. of Molecular Biology and Genetics, The Johns Hopkins University.

pPC97 is a yeast low copy shuttle vector, encoding GAL4 DNA binding domain (DBD), amino acids 1-147, expressed from the strong constitutive promoter for yeast alcohol dehydrogenase (ADH1) gene. It contains LEU2 as a selectable marker in yeast, as well as an autonomously replicating sequence (ARS) and centromere (CEN) sequence for stable replication and segregation of the plasmid at cell division.

pPC86 is a similar yeast low copy shuttle vector coding for GAL4 activation domain (AD), amino acids 768-881, expressed from the constitutive ADH1 promoter. It carries TRP1 as a selectable marker in yeast.

pMA424 (Ma and Ptashne, 1987), was obtained from Dr. Stanley Fields, Dept. of Microbiology, S.U.N.Y. at Stony Brook. **pMA424** is a yeast high copy shuttle vector, coding for GAL4 DBD expressed from the constitutive ADH1 promoter. It contains HIS3 as a selectable marker in yeast and 2 μ sequence for replication and maintaining high copy number of the plasmid in yeast.

2.1.6. Cloning vectors

pSPUTK-65 contains the entire coding sequence for VP16 (490 amino acids) cloned between Nco I and Bam HI sites of pSPUTK (Falcone and Andrews, 1991).

pSPUTK-65B is constructed by Dr. J. Capone and represents a Bam HI collapse of pSPUTK-65 after insertion of a Bam HI linker into Sac II site of the coding sequence of VP16. The resulting plasmid contains the coding sequence of the amino terminal 404 amino acids of VP16.

pSP-T379, pSP-T369, pSP-T335, pSP-T299, pSP-T250, pSP-Δ141-178, pSP-Δ25-178 code for the corresponding carboxy-terminal deletion or internal in-frame deletion mutants of VP16, subcloned from previously described plasmids (Werstuck and Capone, 1989a) into **pSPUTK** by Rob Wheatley.

pSPAS is constructed by C. Smibert and contains a fragment of the coding sequence of vhs (amino acids 179-344) cloned between *Apa* I and *Sma* I sites of **pSPUTK**.

2.1.7. Mammalian plasmids

pSG424 codes for GAL4 DBD (amino acids 1 to 147), expressed from SV40 early promoter (Sadowski and Ptashne, 1989).

pGAL4-VP16_{ΔAD} expresses GAL4 DBD fused in-frame to the 78 carboxy-terminal amino acids of VP16 and is identical to **pSGVP** (Sadowski *et al.*, 1988).

pGAL4₅/E1bCAT contains five copies of the cognate GAL4 DNA binding site upstream of the *E.coli* chloramphenicol acetyl transferase (CAT) gene (Lillie and Green, 1989).

2.1.8. Yeast strain and growth media

The *Saccharomyces cerevisiae* strain PCY2 (Chevray and Nathans, 1992) was a generous gift from Dr. P. Chevray. The genotype of PCY2 is: *MATα Δgal4 Δgal80 URA3::GAL1-lacZ lys2-801^{amber} his3-Δ200 trp-Δ63 leu2 ade2-101^{ochre}*.

Yeast were grown at 30°C in rich medium containing 1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose (YEPD). Synthetic complete (Sc) medium containing 0.67% yeast nitrogen base, 2% dextrose and 0.2% of amino acid mix lacking the appropriate amino acid, was used to select for the corresponding transformants (Ausbel *et al.*, 1990).

2.1.9. Bacterial strain and growth media

Bacterial strain used for transformations and plasmids amplification was *E.coli* DH5 α purchased from GIBCO BRL.

For plasmid isolation cells were grown at 37°C in 2xYT medium (1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl) containing 100 $\mu\text{g ml}^{-1}$ ampicillin (Sambrook *et al.*, 1989).

2.1.10. Oligonucleotides

The oligonucleotide 5'-GAG AGT AGT AAC AAA GGT C-3', used for DNA sequencing, was obtained from the Central Facility of the Institute for Molecular Biology (MOBIX), McMaster University. This sequence is identical to the coding strand of GAL4-DBD specifying codons 140 to 145.

2.2. Isolation of plasmid DNA

2.2.1. Small scale plasmid isolation

Plasmid DNA was isolated by the rapid boiling method (Sambrook *et al.*, 1989) from 1.5 ml saturated cultures grown in 2xYT medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin (2xYT/amp). The cells were collected by centrifugation and resuspended in 0.35 ml of miniprep lysis buffer (0.01 M Tris-Cl pH 8.0, 8% sucrose, 0.05 M EDTA, 0.5% Triton X-100). After addition of 0.03 ml of freshly prepared lysozyme solution (10 mg ml^{-1}), the cells were vortexed vigorously and incubated for 60 s in a boiling water bath. The tubes were centrifuged for 15 min at 4°C in a microfuge and the resulting pellet was removed immediately with a toothpick. Plasmid DNA was precipitated by addition to the supernatant 0.2 ml of 7.5 M NH_4Ac and 0.7 ml of iso-propanol, and incubation for at least 30 min at -20°C. Precipitated plasmid DNA was centrifuged in a microfuge for 15 min at 4°C. The obtained pellet was washed with 70% EtOH, dried for 5-10 min at RT, and finally resuspended in 0.05 ml of TE (pH 8.0) containing 20 $\mu\text{g ml}^{-1}$ of RNase A.

2.2.2. Large scale isolation

Plasmid DNA was isolated from 250 ml saturated cultures, grown in 2xYT/amp, using QIAGEN-tip500 columns, according to the instructions of the manufacturer. QIAGEN purification protocol is based on the alkaline lysis method combined with chromatographic separation aimed to selectively isolate plasmid DNA from RNA, proteins and other cellular contaminants present in the lysate. Briefly, after lysing the

cells with NaOH/SDS solution, the lysate was neutralised with acidic potassium acetate, which causes precipitation of the denatured proteins and chromosomal DNA, while the smaller plasmid DNA renature rapidly. After removing the cell debris by high speed centrifugation, the cleared lysate, containing mainly plasmid DNA, was applied to a QIAGEN-tip500 column. While plasmid DNA selectively binds to the anion-exchange silicagel resin, most of the contaminants are not retained or are eluted during the successive washes of the column. After elution with high salt concentration buffer, plasmid DNA was precipitated with iso-propanol, washed with 70% EtOH and resuspended in TE (pH 8.0).

2.3. DNA manipulation

2.3.1. Determination of DNA concentration

DNA concentration was determined measuring UV absorbance of the samples at 260 nm on Beckman DU64 Spectrophotometer (Ausbel *et al.*, 1990).

2.3.2. Digestion with restriction enzymes

Digestion of DNA with restriction enzymes was performed under conditions recommended by the manufacturer.

2.3.3. Filling-in recessed 3' termini

Recessed 3' ends were filled in using 1 to 5 units of Klenow fragment of *E.coli* DNA polymerase I (Sambrook *et al.*, 1989). The final concentration of the added dNTPs was 0.05 mM each and the reaction was carried out at room temperature (RT) for 30 min. The enzyme was phenol-chloroform extracted and DNA precipitated as described in 2.3.6.

2.3.4. Digestion of protruding 5' termini

Mung bean nuclease was used for digestion of protruding 5' ends. The reaction was carried out in 0.03 M sodium acetate buffer (pH 4.5), 0.05 M NaCl, 0.01 M ZnCl₂, 5% glycerol (Sambrook *et al.*, 1989). The samples were incubated for 30 min at 37°C using approximately 5 U of the enzyme per µg DNA. The enzyme was heat inactivated, DNA precipitated and purified from agarose gel.

2.3.5. Dephosphorylation of 5'-termini

Dephosphorylation of 5'-ends, in order to prevent self-ligation of the vector, was performed using calf intestine phosphatase (CIP). 1 U of the enzyme was used per 10 µg DNA in a buffer supplied by the manufacturer (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM MgCl₂, 1.0 mM DTT). The reaction was carried out at 37°C for 30 min, followed by second incubation at 50°C. The enzyme was heat inactivated for 10 min at 75°C in the presence of 5.0 mM EDTA, DNA was precipitated and gel purified.

2.3.6. DNA precipitation

Precipitation of DNA was performed in the presence of 2.5 M ammonium acetate (Sambrook *et al.*, 1989). After addition of 0.5 volume of 7.5 M NH_4Ac to the sample, DNA was precipitated with an equal volume of iso-propanol for at least 30 min at -20°C . DNA was then centrifuged in a microfuge for 15 min at 4°C , washed with 70% EtOH and resuspended in TE (pH 8.0).

2.3.7. DNA purification

DNA fragments were separated by gel electrophoresis using 1% NuSieve low melting point (LMP) agarose. The fragment of interest was cut out from the gel and transferred to a microfuge tube containing two volumes of 0.2 M Tris-HCl pH 8.0. The sample was heated at 65°C until the agarose melted (5-10 min). The solution was then extracted first with an equal volume of Tris-saturated phenol and with 0.2 ml of sterile H_2O . Both aqueous phases were combined and two successive extractions with phenol-chloroform-iso-amyl alcohol (25:25:1) were performed, followed by a chloroform extraction. One-tenth volume of 3.0 M sodium acetate (pH 5.2) was then added to thus obtained aqueous phase and DNA was precipitated with an equal volume of iso-propanol for > 30 min at -20°C . DNA was centrifuged in a microfuge, rinsed with 70% EtOH and resuspended in a small volume of TE (pH 8.0).

2.3.8. DNA ligation

DNA ligations were performed using T4 DNA ligase (NEB) in a buffer supplied by the manufacturer (50 mM Tris-HCl pH 7.8; 10 mM MgCl_2 ; 10 mM DTT; 1.0 mM ATP). One-tenth to 1.0 μg of vector DNA was incubated with the corresponding insert overnight at 16°C in 10 to 15 μl total volume reactions.

2.3.9. DNA sequencing

DNA sequencing was performed using Sequenase Version 2.0 Kit (United States Biochemicals) according to the protocol of the manufacturer. Ten μg of plasmid DNA were denatured in 0.2 M NaOH 0.2 M EDTA for 5 min at 37°C. The sample was then placed on ice to prevent reannealing and 3.0 M sodium acetate was added until the pH was brought up to 7.0. DNA was precipitated with two volumes of EtOH and resuspended in TE. One pmol of the synthetic oligonucleotide was added to 3-5 μg of thus prepared single stranded DNA, heated for 2 min at 65°C and cooled slowly for at least 30 min to allow annealing of the primer to the DNA template. The labelling reaction was performed for 2 min (instead of 5 min), using 0.5 μM dNTPs each (including [α - ^{32}P]dATP) instead of 1.5 μM dNTPs as described in the protocol of manufacturer. The termination of chain synthesis with the corresponding ddNTP was performed in the presence of Mn^{2+} . Mn^{2+} reduces the ability of the Sequenase to distinguish between dNTPs and ddNTPs thus increasing the extend of incorporation of ddNTPs and therefore the termination of the complementary chain synthesis. As a result,

under these conditions, nucleotide sequence close to the primer (approximately 20 nucleotides) can be obtained (Ausbel *et al.*, 1990). After termination of the reactions by addition of EDTA and formamide, DNA was denatured by heating at 80°C for 2 min. The DNA fragments were separated by electrophoresis on 8% acrylamide 7.0 M urea sequencing gel at 100 watts. The gels were exposed to Kodak X-Omat K film.

2.4. Transformation of *E.coli*

Subcloning efficiency DH5 α competent cells (GIBCO BRL) were used for transformations, performed according to the instructions of the manufacturer. Approximately 0.01 to 1 μ g transforming DNA in less than 5 μ l of total volume was mixed with 50 to 100 μ l of competent cells and incubated on ice for 30 min. The cells were heat shocked for 30 s at 37°C and 0.9 ml of 2xYT medium was added to each sample. The cells were grown for 1 h at 37°C with constant agitation, to allow expression of the β -lactamase gene present on the plasmid. Fifty to 100 μ l aliquots were plated on selective medium (2xYT/amp). Ampicillin resistant colonies appeared after overnight incubation of the plates at 37°C.

2.5. Transformation of yeast

2.5.1. High efficiency transformation of yeast using lithium acetate (LiAc) method

Transformation of yeast was performed with some modifications of the method described by Schiestl and Gietz (1989). The procedure is based on the observation that

treatment with alkali cations make yeast able to uptake foreign DNA. A short incubation of yeast in LiAc solution, followed by addition of polyethylene glycol (PEG) and heat shock at 42°C, induces DNA uptake. Briefly, the night before transformation 5 ml of yeast saturated culture was transferred to 250 ml YEPD and grown overnight at 30°C. The overnight culture was diluted into 250 ml YEPD containing 30 mg ml⁻¹ adenine (YPAD) so that OD₆₀₀ was between 0.3 and 0.5. Addition of adenine to YEPD has been shown to increase the efficiency of transformation, especially for *ade⁻* strains (Ausbel *et al.*, 1990). The yeast were grown in YPAD for 2 to 4 h until mid-log phase (OD₆₀₀ between 0.6-0.8). The cells were harvested by centrifugation at 5000xg (Sorvall GSA rotor) for 5 min and gently washed in 10 ml of sterile water. After transferring the suspension in 40 ml tubes, the cells were centrifuged at 7000xg (Sorvall SS34 rotor) for 5 min. The yeast were resuspended in 1.5 ml of sterile 1xLiAc/TE solution prepared fresh from 10x filter sterilized stocks (10xLiAc is 1.0 M LiAc pH 7.5 adjusted with dilute acetic acid; 10xTE is 0.1 M Tris-HCl pH 7.5, 0.01 M EDTA). Incubation of the yeast suspension at 30°C, as well as the following sonication of the cells (recommended in the original protocol) were not performed, as it was found that these steps did not increase the efficiency of transformation. Up to 5 µg of plasmid DNA was mixed with 100 µg of sonicated and denatured salmon testes DNA (SIGMA) in a round bottom Falcon tube (microfuge tubes were not used as during the incubation of yeast with PEG the cells settled down). The total volume of plasmid and carrier DNA did not exceed 20 µl. The DNA was gently mixed with 200 µl of the yeast suspension in 1xLiAc/TE and

the cells were incubated for 30 min at 30°C with agitation. The cells were carefully mixed with 1.2 ml of sterile PEG solution (40% PEG 3350, 1xLiAc, 1xTE) prepared fresh from filtered stocks (50% PEG 3350; 10xLiAc; 10xTE) and the incubation was continued for another 30 min. The yeast were then heat shocked for 15 min at 42°C, transferred to a microfuge tube and pelleted. The cells were resuspended using sterile toothpick in 1xTE, spun down and washed again in 1xTE. The yeast were resuspended in 1xTE and plated out on selective medium. The plates were incubated at 30°C and the colonies appeared 2 to 5 days after transformation.

2.5.2. A simplified LiAc/PEG protocol for transformation of yeast

Number of transformants obtained with the simplified LiAc/PEG procedure was sufficient for the most purposes when high efficiency of transformation was not required for a particular experiment. The advantages of this method are the greatly reduced labour and number of manipulations per sample, as well as an independence from yeast growth phase. The protocol is based on an extended incubation of yeast and DNA in the presence of LiAc/PEG solution, and was performed as described by Elble (1992). Briefly, 1 ml of yeast culture grown in YEPD or YPAD, was centrifuged in a microfuge tube and the supernatant was discarded. One μg of plasmid DNA and 100 μg of salmon testes DNA (SIGMA) were added to the yeast pellet and mixed by vortexing for 2-3 s. A half ml of sterile PLATE solution was added to the tube (PLATE: 40% PEG 3350; 0.1 M LiAc pH 7.5; 0.01 M Tris-HCl pH 7.5, 0.001 M EDTA. The 10x stock solutions

used for the high efficiency LiAc transformation method, can be used to prepare PLATE. The solution does not have to be freshly prepared and can be stored at 4°C). The tube was vortexed again to mix the PLATE solution with the yeast and DNA, and incubated overnight at RT. Approximately 50 μ l of the settled cells were plated on selective medium. Transformants appeared after 3-5 days incubation at 30°C.

2.6. β -galactosidase assay

2.6.1. Colony filter assay for qualitative determination of β -galactosidase activity

The assay was used as a rapid screen for identifying yeast transformants with β -galactosidase activity. The advantages of the method are its high sensitivity and the possibility to screen thousands of colonies at the same time directly from plates. The assay was performed with some modification of the procedure described by Breedon *et al.* (1985). Yeast colonies grown on plates were replica-transferred to NYTRAN filters which were then dipped in liquid nitrogen for 3-5 s to permeabilize the cells. The filters were then thawed at RT. A disk of Whatman 1 paper was placed in a petri plate and saturated with 1.5 ml of buffer Z (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1.0 mM MgSO₄, 50 mM β -mercaptoethanol, pH 7.0), containing 1 mg ml⁻¹ of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal). The filters with the attached permeabilized colonies were placed over the Whatman discs. The plates were incubated at 37°C until the blue colour, developed due to X-Gal cleavage, was detected (30 min to overnight).

2.6.2. Quantitative determination of β -galactosidase activity

β -galactosidase activity of individual transformants grown in liquid cultures was performed using o-nitrophenyl- β -D-galactoside (ONPG) as a substrate. After cell permeabilization with 0.1% SDS and chloroform, and incubation with the substrate, the intensity of the developed yellow colour was measured spectrophotometrically. The units of β -galactosidase activity were normalized for cell density and the time of incubation. The assay was performed as described by Ausbel *et al.* (1990). Five ml yeast cultures grown overnight at 30°C were collected in a clinical centrifuge. The cells were resuspended in buffer Z (see above), placed on ice and OD₆₀₀ was determined for each sample. Usually, 100 μ l of cell suspension with OD₆₀₀ of about 0.5 were transferred to microfuge tubes and the total volume was brought up to 1 ml with buffer Z. Ten μ l of 0.1% SDS and 20 μ l of chloroform were added to each sample and the tubes were vortexed vigorously for 15 s. The samples were then incubated for 15 min at 30°C. After addition of 0.2 ml of 4 mg ml⁻¹ ONPG to each tube, the samples were vortexed briefly and incubated at 30°C. The reaction was stopped with 0.5 ml of 1.0 M Na₂CO₃ when yellow colour had developed and the duration of the incubation was recorded. The cells were centrifuged for 5 min in a microfuge. The OD at 420 nm and 550 nm of the supernatant was measured. β -galactosidase activity was calculated according to the equation (Ausbel *et al.*, 1990):

$$U = 1000 * \frac{[(OD_{420}) - (1.75 * OD_{550})]}{(t) * (v) * (OD_{600})}$$

Where:

- OD₄₂₀ - absorbance by o-nitrophenol and light scattering by the cells
- OD₅₅₀ - light scattering by the cells
- t - time of incubation (min)
- v - volume of the culture used in the assay (ml)
- OD₆₀₀ - cell density of the culture used in the assay

2.7. Analysis of proteins

2.7.1. Protein determination by BioRad assay

Protein concentration was determined using BioRad assay which is based on the method developed by Bradford (1976). After addition of 0.2 ml of the Dye reagent concentrate (Coomassie Brilliant Blue G-250) to 0.8 ml of the protein samples (up to 10 µg), the absorbance at 595 nm was measured. The protein content was determined by interpolating the values received for the samples to a standard curve prepared at the same time (bovine albumin was used as a standard).

2.7.2. Lysing yeast cells for immunoblot analysis of the proteins

Yeast cells were lysed using glass beads disruption method essentially as described by Ausbel *et al.* (1990). Five ml of saturated yeast culture was pelleted in a clinical

centrifuge and the cells were washed in an equal volume of PBS. After removing the supernatant, the yeast were resuspended in a small volume of ice-cold lysis buffer (RIPA: 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl pH 8.0, 1.0 mM PMSF added before use from 100 mM stock solution). All subsequent manipulations were performed at 4°C. The cells were transferred to microfuge tubes, centrifuged for 2-3 s and resuspended in 200 μ l RIPA. An equal volume of chilled glass beads was added to the samples. The tubes were vortexed vigorously for 30 s and chilled afterwards on ice for 30-60 s. This step was performed 4-5 times. After addition of 100 μ l of ice-cold RIPA to the samples, the tubes were vortexed once again for 30 s. Thus, the cells were vortexed for at least 2-2.5 min total. The glass beads and the cell debris were removed by centrifugation for 5 min at highest speed. The supernatant was aliquoted in new tubes and stored at -20°C. The protein content of the lysates, after diluting the samples, was determined as described above.

2.7.3. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

Separation of the proteins according to their size was performed using SDS PAGE as described by Laemmli (1970). The separating gel was 10% acrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.1% v/v TEMED (N,N,N',N'-tetramethylene-diamine), 0.1% ammonium persulphate (APS). The stacking gel was 4% acrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.1% v/v TEMED, 0.1% APS (Sambrook, 1989). The gels were poured and run in a BioRad vertical slab mini-gel apparatus. The samples were mixed

with an equal volume of 2x sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.2% bromophenol blue) and heated for 5 min in a 100°C water bath. The gels were run in SDS PAGE running buffer (25 mM Tris-HCl pH 8.3, 250 mM glycine, 0.1% SDS) at 100-150 V.

2.7.4. Immunoblot analysis of the proteins

Proteins were separated by SDS PAGE and transferred to nitrocellulose paper using BioRad transblot apparatus. The chamber was filled with transfer buffer (150 mM glycine, 20 mM Tris-HCl pH 8.3, 20% methanol) and the transfer was accomplished for 12-16 h at 70-80 mA. The subsequent treatment of the filters was performed according to the ECL Western blotting protocol (Amersham). All procedures were performed at RT. After completing the transfer, the filter was blocked in 5% low fat dry milk dissolved in Tris-buffered saline - Tween (TBST: 20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20). The incubation was performed for 1 h with rotation on an orbital shaker. The filter was then washed in TBST (the membrane was rinsed twice and incubated once for 15 min and two times for 5 min with fresh changes of the washing buffer). The filter was then transferred to a plastic pouch (Kapak Corp., Minneapolis, MN) and the bag was heat sealed after addition of 5 ml of the primary antibody (rabbit anti-GAL4 antiserum diluted 2000x in TBST). The incubation was performed for 60 min on a platform rocker. The filter was removed and washed in TBST as described above. The membrane was then incubated with the secondary antibody (anti-rabbit Ig,

horseradish peroxidase linked whole antibody, diluted 30000x in TBST) for 1 h on an orbital shaker. The filters were then washed in TBST (rinsed twice as above and incubated once for 15 min and 4 times for 5 min in fresh changes of the washing buffer). The antigen was detected using the enhanced chemiluminescence (ECL) detection system. The principle of the system is based on the oxidation of luminol by HRP in the presence of chemical enhancers. The oxidized luminol is in an excited state and emits light at 428 nm which can be detected by an exposure to autoradiography film. The advantages of this system are the high sensitivity (about 10 times higher than the other non- and radioactive methods) and the speed of detection (as proteins can be detected in less than 1 min). Briefly, the filters were drained from the excess TBST and incubated for 1 min in equal mixture of reagent 1 and 2, so that the surface of the membrane is evenly covered. The reagent mixture was then drained off, the membrane covered with Saran wrap and immediately exposed to film. Usually, exposure between 10 - 45 s was sufficient to visualize the proteins.

2.8. Yeast aggregation assay

The dependence of yeast aggregation phenotype on the presence of Ca^{2+} was tested in order to distinguish between sexual agglutination and cell flocculation. Sexual agglutination is Ca^{2+} independent and is not affected by addition of EDTA to the yeast culture. On the contrary, Ca^{2+} is necessary for flocculation and the clumps are disrupted by addition of EDTA which depletes the available Ca^{2+} . Addition of excess Ca^{2+} to the

EDTA treated culture leads to the formation of the clumps again. The assay was performed as described by Kramer *et al.* (1990). Five ml cultures were grown overnight at 30°C in selective medium and 0.2 ml of 0.5 M EDTA was then added to each culture, so that the final concentration of EDTA was 20 mM. (A disruption of the clumps was observed). After addition of 0.15 ml of 1.0 M CaCl₂ the EDTA treated culture, so that the final concentration of Ca²⁺ was 10 mM above EDTA, and shaking the culture, the clumps formed again.

2.9. Transfection of mammalian cells

Transfections were performed with some modifications of the procedure described by Graham and Van der Eb (1973). COS-1 (SV40 transformed African Green Monkey) cells were maintained in Dulbecco modified essential medium, supplemented with 10% calf serum, 10% penicillin/streptomycin and 1% glutamine (DME complete). Six cm dishes were seeded with 1.5 ml of COS-1 cells from confluent 10 cm dish 24 h before the experiment, so that sub-confluent cultures were used in transfections. Plasmid DNA was mixed with salmon sperm DNA to make the total DNA concentration 20 µg per dish. After addition of 31 µl of 2.0 M CaCl₂ to the DNA, the volume was made up to 0.25 ml with sterile water. This mixture was added to a tube containing 0.25 ml of 2xHBS (1xHBS is 0.05 M HEPES, 1.5 mM sodium phosphate pH 7.12, 0.28 M NaCl). The tube was vortexed at low speed while adding the drops of DNA/CaCl₂ solution. The final mixture, containing 0.125 mM CaCl₂, was incubated for 20 min at RT during which

the calcium phosphate/DNA co-precipitate was formed. The cells were washed with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 , pH 7.3) and 5 ml of DME complete medium was added to each dish. The DNA precipitate was added drop-wise to the plates and cells were incubated for 4 h at 37°C. The medium was then removed and the cells were shocked for 1 min at RT with DME containing 10% dimethyl sulfoxide. The medium was aspirated and the cells were washed three times in DME. Five ml of DME complete medium was added to each plate and the cells were incubated at 37°C for 48 h.

2.10. Preparation of cell extracts for chloramphenicol acetyl transferase (CAT) assay

After incubation of the transfected cells for 48 h at 37°C, the medium was aspirated and the cells were washed three times with PBS. One ml of TEN buffer (40 mM Tris-HCl pH 7.4, 1.0 mM EDTA, 0.15 M NaCl) was added to each dish and the plates were incubated for 5 min at RT. All subsequent manipulations were performed at 4°C. The cells were scraped with a cell lifter and transferred to microfuge tubes. The cells were centrifuged for 2 min and, after discarding the supernatant, suspended in 100 μl of CAT assay buffer (250 mM Tris-HCl pH 7.8, 0.5% NP40, 1.0 mM PMSF). The cells were lysed by vortexing and sonication (three times for 10 s each) and the cell debris were discarded after centrifugation in a microfuge for 5 min at the highest speed. The supernatant was transferred to new tubes and the protein content was determined

using BioRad assay.

2.11. Chloramphenicol acetyl transferase assay

Chloramphenicol acetyl transferase is a bacterial enzyme catalyzing transfer of an acetyl group from acetyl coenzyme A (CoA) to chloramphenicol, thus inactivating the antibiotic. The principle of the assay is based on incubation of cellular extracts containing CAT activity with radiolabelled chloramphenicol in the presence of acetyl CoA. After organic extraction, the acetylated products are separated from the unmetabolized chloramphenicol by thin layer chromatography and the results are detected by autoradiography. The protocol used is a modification of the procedure described by Gorman *et al.* (1982). One μl of [^{14}C]-chloramphenicol, 25 μl of 5.0 mM acetyl CoA (freshly prepared) and 70 μl of 1.0 M Tris-HCl pH 7.8 were added to approximately 20 μl of cell extracts (normalized for protein content). The total volume was brought up to 150 μl with sterile H_2O , the tube was vortexed and incubated for 1 h at 37°C. One ml of ethyl acetate was then added to the reaction and after vortexing and centrifuging for 2 min, the top organic phase, containing chloramphenicol and its acetylated products, was carefully transferred to a new microfuge tube. After drying down in a Speed Vac for 1 h, the pellet was resuspended in 25 μl of ethyl acetate, vortexed and spotted on pre-coated silica gel plates (Whatman). The chromatogram was developed in chloroform/methanol system (95:5, ascending), air dried and exposed to film overnight at RT.

RESULTS

Section I

3. VP16 interacts with vhs *in vivo*

Vhs, like VP16, is a HSV-1 late phosphoprotein located in the virion tegument (Fenwick and Everett, 1990; Smibert *et al.*, 1992; McLauchlan *et al.*, 1992). Unlike VP16, it is much less abundant in both virions and infected cells (McLauchlan *et al.*, 1992) and it is not essential for virion particle assembly (Fenwick and Everett, 1990; Smibert and Smiley, 1990). Vhs function is involved in suppression of host protein synthesis (vhs stands for virion-induced host shutoff) and acceleration of cellular and viral mRNA turnover (Kwong and Frenkel, 1987; Kwong *et al.*, 1988; Fenwick and Everett, 1990; Smibert and Smiley, 1990). Vhs binds to VP16 *in vitro*, as shown by co-precipitation of VP16 from HSV-1 infected cell extracts with anti-vhs antiserum (Smibert *et al.*, 1993). The interaction between the two viral proteins was confirmed by the ability of VP16-proteinA fusion protein, covalently immobilized on Sepharose beads, to retain specifically *in vitro* translated vhs (solid-phase capture assay), and *vice-versa* (Smibert *et al.*, 1993). Mutational analysis employed to determine the region of vhs involved in the complex formation revealed that residues 238 to 344 of the vhs polypeptide are necessary and sufficient for binding to VP16 in the solid-phase capture

assay (Smibert *et al.*, 1993). Similar analysis of VP16 showed that although the acidic activation domain is completely dispensable, a relatively large portion of the protein encompassing the amino terminal 369 amino acids is necessary for this interaction to occur *in vitro* (Smibert *et al.*, 1993).

Using the two-hybrid system, detection of β -galactosidase activity in yeast expressing the binding domain of vhs and VP16 (lacking the activation domain) fused to GAL4-AD and GAL4-DBD, respectively, would indicate that the interaction between these proteins occurs *in vivo* as well. In addition, it would establish the feasibility of utilizing the system for identification of cellular proteins interacting with VP16.

3.1. Analysis of the interaction between VP16 and vhs in the yeast two-hybrid system

3.1.1. Cloning VP16 (amino acids 1-404) into GAL4-DBD fusion vector

The coding sequence for the N-terminal 404 amino acids of VP16 (VP16₄₀₄), was obtained from pSPUTK-65B (contains the entire VP16 ORF) after linearizing with Nco I (specifying codon 1), blunt ending with Klenow polymerase and digestion with Bam HI (present as a linker insertion at codon 404). Thus obtained VP16₄₀₄ insert was ligated to the compatible ends of the Sma I/Bgl II cut GAL4-DBD fusion vector pPC97 (see Materials and Methods) to construct pCDBVP16₄₀₄. pCDBVP16₄₀₄ (Fig.1) codes for

GAL4-DBD fused upstream of the amino terminal 404 residues of VP16. Sma I, Eco RI and Bgl II sites (present in the MCS of pPC97) are destroyed, while the Nco I site (at codon 1 of VP16) is preserved, as a result of the cloning procedure (Fig.1).

3.1.2. Cloning vhs (amino acids 179-344) into GAL4-AD fusion vector

Vhs coding sequence for amino acids 179-344, was obtained from pSPAS (see Materials and Methods) after linearizing at the Nco I site, present just before vhs ORF, blunt ending with Klenow polymerase and digestion with Bgl II (in the MCS of the plasmid). Thus obtained vhs insert was ligated to the compatible ends of Sma I/Bgl II cut GAL4-AD fusion vector pPC86 (see Materials and Methods) to generate pCADvhs. pCADvhs (Fig.2) directs expression of a hybrid protein consisting of GAL4-AD fused upstream of vhs residues 179 to 344. Nco I and Bgl II sites (present at the 5' and 3' ends, respectively, of vhs) are preserved, while Sma I and Eco RI sites (in the MCS of pPC86) are destroyed, as a result of the cloning procedure (Fig.2).

3.1.3. Analysis of the interaction of the hybrid proteins *in vivo*

In order to assay the interaction between VP16 and vhs *in vivo*, the described above plasmids were introduced into the yeast strain PCY2($\Delta gal4 \Delta gal80$ $URA3::GAL1-lacZ$ $trp1-\Delta 63$ $leu2$) using the lithium acetate (LiAc) transformation protocol. Yeast transformed with both GAL4-DBD and GAL4-AD fusion vectors were selected by plating out on synthetic complete medium lacking Leu and Trp (Sc-Leu-Trp) which are

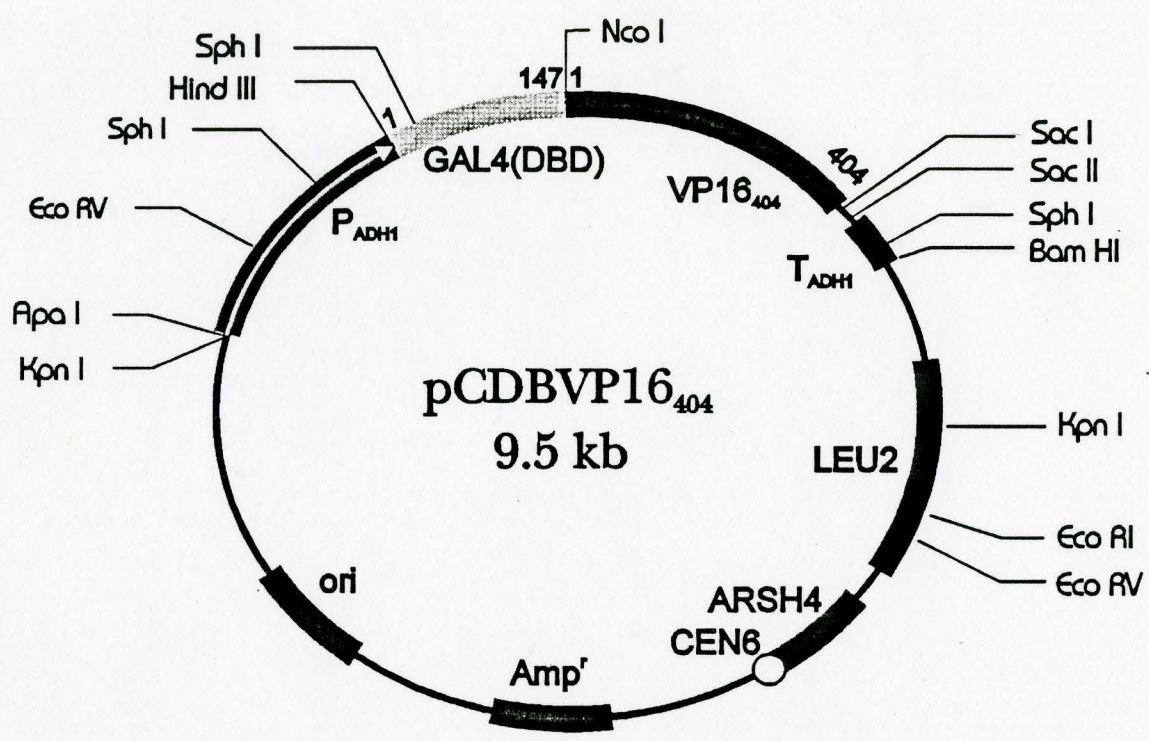


Fig. 1 pCDBVP16₄₀₄ is a yeast low copy shuttle vector, which directs expression of GAL4-DBD (amino acids 1-147) fused upstream of VP16 404 amino terminal residues. It contains the following functional elements: a constitutive promoter (P_{ADHI}) and a terminator (T_{ADHI}) for the yeast alcohol dehydrogenase gene; LEU2 as selectable marker in yeast; ARS/CEN sequence for stable replication and segregation in yeast; ampicillin resistance gene (Amp^r) and bacterial origin of replication (ori). (Adapted from Chevray and Nathans, 1992).

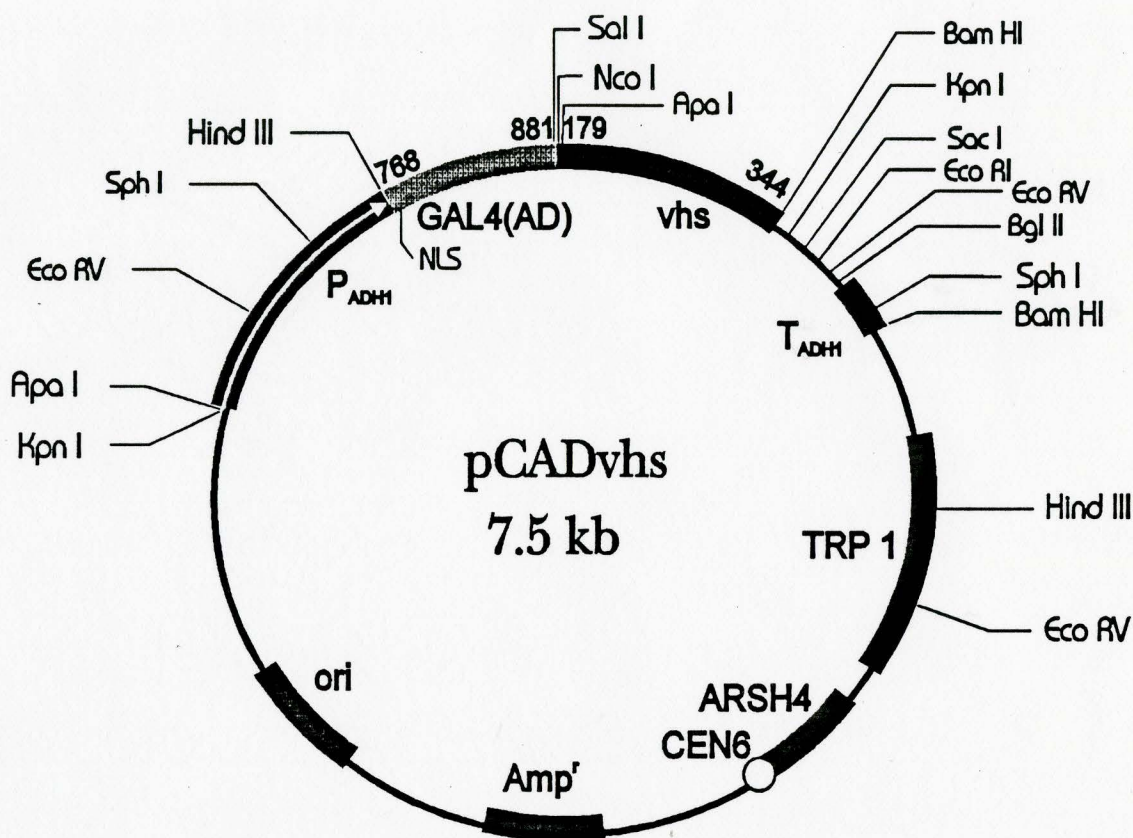


Fig. 2 pCADvhs is a yeast low copy shuttle vector, which directs expression of GAL4-AD (amino acids 768-881) fused upstream of vhs 179-344 amino acids. It contains the following functional elements: a constitutive promoter (P_{ADHI}) and a terminator (T_{ADHI}) for the yeast alcohol dehydrogenase gene; TRP1 as selectable marker in yeast; ARS/CEN sequence for stable replication and segregation in yeast; ampicillin resistance gene (Amp^r) and bacterial origin of replication (ori). (Adapted from Chevray and Nathans, 1992).

the markers present on the corresponding plasmids. Yeast transformed with a singular expression vector were selected on Sc-Leu or Sc-Trp, respectively. An interaction between the hybrid proteins in the double transformants would bring GAL4-AD to GAL4-DBD restoring the activation function of GAL4. Thus, the expression of the regulated by GAL4-responsive promoter, resident lacZ gene, would be stimulated and detection of β -galactosidase activity would indicate an interaction between the hybrid proteins. β -galactosidase activity of the obtained transformants was determined directly from the plates using the qualitative yeast colony filter assay with X-Gal as a substrate. Individual transformants were then grown in liquid cultures in selective medium for the plasmid and β -galactosidase activity was determined quantitatively using ONPG as a substrate. The obtained results are summarized in Table 1. Neither GAL4 DNA binding domain nor GAL4 activation domain (alone or in combination) transactivated the lacZ reporter gene. As expected, vhs fused to GAL4 activation domain did not stimulate the expression of β -galactosidase, either. Surprisingly, VP16₄₀₄ fused to GAL4 DNA binding domain was alone sufficient for transactivation of the reporter gene. (This phenomenon is explored in Section II). β -galactosidase activity of the yeast transformed with both fusion proteins (VP16₄₀₄ and vhs) and VP16₄₀₄ alone, was essentially identical (150 ± 11 and 142 ± 6 , respectively). Thus, it was not possible to conclude if the interaction between VP16 and vhs *in vivo* indeed occurred. As the transactivation caused by VP16₄₀₄ was most likely dependent on its binding to the cognate DNA sequence through GAL4 DNA binding domain, it was speculated that fusion of VP16₄₀₄ to the GAL4 activation

Table 1:

Yeast transformed with:		β -galactosidase activity	
GAL4-DBD hybrid	GAL4-AD hybrid	filters (colour)	liquid cultures (U)
DBD	-	white	< 1
-	AD	white	< 1
DBD	AD	white	< 1
DBD-VP16 ₄₀₄	-	blue	142 \pm 6
-	AD-vhs	white	< 1
DBD-VP16 ₄₀₄	AD-vhs	blue	150 \pm 11

The presented values of β -galactosidase activity are the averages of at least five independent transformants assayed in duplicate and normalized to cell density (OD₆₀₀). The standard errors are indicated.

domain would not result in activated expression of the reporter gene. Thus, testing vhs fused to GAL4-DBD and VP16₄₀₄ to GAL4-AD in the same system, would answer the question about the interaction between these proteins *in vivo*.

3.1.4. Cloning VP16 (amino acids 1-404) into GAL4-AD fusion vector

The coding sequence for the amino-terminal 404 residues of VP16 was obtained as described in 3.1.1. and ligated to the compatible ends of Sma I/Bgl II digested GAL4-AD fusion vector pPC86 to generate pCADVP16₄₀₄. pCADVP16₄₀₄ (Fig.3a) directs expression of the amino terminal 404 residues of VP16 fused downstream of GAL4 activation domain. Sma I, Eco RI and Bgl II sites (present in the MCS of pPC86) are destroyed, while the Nco I site (at codon 1 of VP16) is preserved, as a result of the cloning procedure (Fig.3a).

3.1.5. Cloning of vhs (amino acids 179-344) into GAL4-DBD fusion vector

vhs coding sequence (codons 179 to 344) was excised from pSPAS as described in 3.1.2. and ligated to the Sma I/Bgl II digested GAL4-DBD fusion vector pPC97 to construct pCDBvhs. pCDBvhs (Fig.3b) encodes for a hybrid protein consisting of GAL4 DNA binding domain fused upstream of vhs residues 179 to 344. Nco I and Bgl II sites (at the 5' and 3' ends, respectively, of vhs) are preserved, while Sma I and Eco RI sites (in the MCS of pPC97) are destroyed as a result of the cloning (Fig.3b).

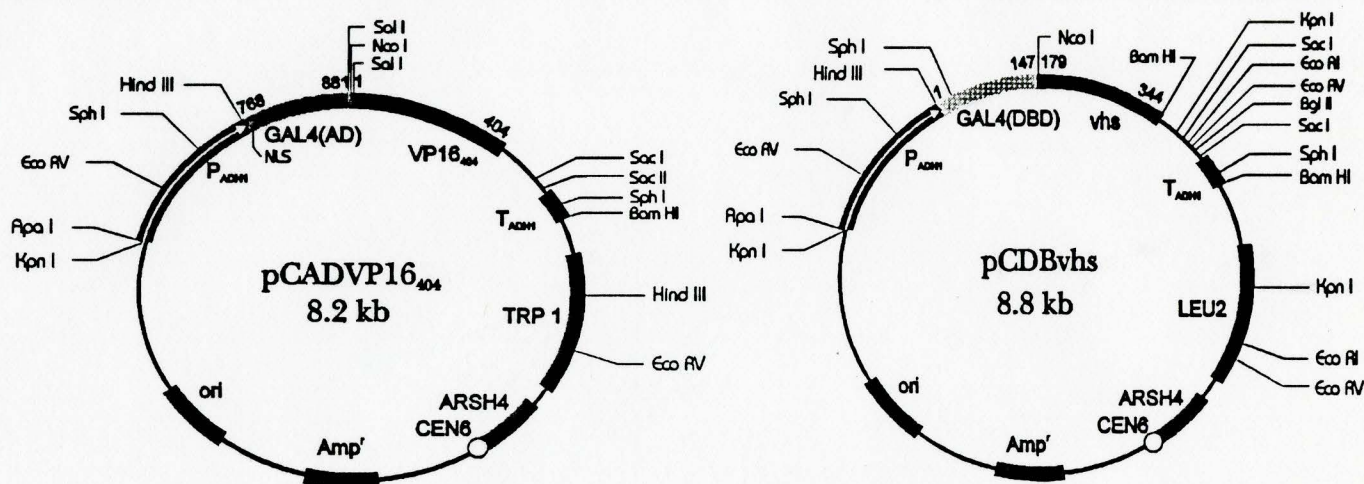


Fig.3 a). pCADVP16₄₀₄ is a yeast low copy number plasmid, expressing GAL4 activation domain fused upstream of VP16 amino terminal 404 residues. It carries TRP1 as selectable marker in yeast.

b). pCDBvhs is a yeast low copy shuttle plasmid which directs expression of a hybrid protein consisting of GAL4 DNA binding domain fused in-frame to vhs residues 179 to 344. It carries LEU2 as selectable marker in yeast.

Both fusion proteins are expressed under control of the strong constitutive promoter for the yeast ADHI gene. In addition, the plasmids carry ARS/CEN sequence for stable replication and segregation in yeast, as well as origin of replication (ori) and Amp^r gene for amplification in *E.coli*. (Adapted from Chevray and Nathans, 1992).

3.1.6. Testing the interaction of the fusion proteins in yeast two-hybrid system

To test the interaction between VP16 and vhs *in vivo* the plasmids coding for GAL4(AD)-VP16₄₀₄ and GAL4(DBD)-vhs fusion proteins were introduced into PCY2 using the simplified LiAc/PEG protocol for transformation of yeast. Single or double transformants were selected on Sc media lacking the appropriate amino acids as described in 3.1.3. β -galactosidase activity of the obtained transformants, which is indicative for protein-protein interaction, was determined qualitatively directly from the plates (colony filter assay) and quantitatively from liquid cultures of individual transformants grown in media selective for the plasmids. The obtained results are summarized in Table 2. As speculated earlier, VP16₄₀₄ fused to GAL4 activation domain was not able to bind to GAL4-specific DNA sequence and transactivate the reporter lacZ gene, as judged by the absence of β -galactosidase activity in yeast transformed with the corresponding plasmid. As vhs does not function as a transcriptional activator, the fragment fused to GAL4 DNA binding domain did not induce the expression of β -galactosidase in yeast, either. GAL4 DNA binding domain and/or GAL4 activation domain were not able to activate the reporter gene, as shown previously. β -galactosidase activity was detected only in yeast expressing both fusion proteins, indicating that interaction of VP16₄₀₄ with vhs reconstituted the proximity of GAL4 activation domain to GAL4 DNA binding domain and activated expression of the reporter lacZ gene driven by GAL4-responsive promoter.

Table 2:

Yeast transformed with:		β -galactosidase activity	
GAL4-DBD hybrid	GAL4-AD hybrid	filters (colour)	liquid cultures (U)
DBD	-	white	< 1
-	AD	white	< 1
DBD	AD	white	< 1
-	AD-VP16 ₄₀₄	white	< 1
DBD-vhs	-	white	< 1
DBD-vhs	AD-VP16 ₄₀₄	blue	86 \pm 8

The presented values of β -galactosidase activity are the averages of at least five independent transformants assayed in duplicate and normalized to cell density (OD₆₀₀). The standard error is indicated.

3.2. Mapping the region of VP16 important for interaction with vhs *in vivo*

The above results clearly demonstrated the interaction between VP16 and vhs *in vivo*. In addition, the system could be used to identify the region of VP16 involved in this function. In order to map VP16 domain necessary for interaction with vhs, a number of carboxy-terminal and internal in-frame deletion mutants of VP16 were fused downstream of GAL4 expression vectors. Their ability to interact with vhs *in vivo* was tested in the two-hybrid system.

3.2.1. Cloning carboxy-terminal deletion mutants of VP16 into GAL4 fusion vectors

pCADVP16₄₂₄ (Fig.4) is a yeast low copy number plasmid, which directs expression of the amino-terminal 424 amino acids of VP16 fused in-frame downstream of GAL4 activation domain. The coding sequence for VP16, used to construct this plasmid, was excised from pSPUTK-65 (contains the entire VP16 ORF) with Nco I (specifying codon 1) and Sac I (at codon 424). The activation domain fusion vector was obtained from pCADVP16₄₀₄ (see Fig.3a) after first cutting out the coding sequence for VP16₄₀₄ with Nco I (at codon 1) and Sac I (present downstream of VP16 in the MCS). The resulting vector, now containing only GAL4-AD sequence, was ligated to the compatible ends of the VP16₄₂₄ insert to generate pCADBP16₄₂₄ (Fig.4).

pCADVP16₃₇₉, pCADVP16₃₆₉ and pCADVP16₃₃₅ (Fig.4) are yeast low copy number plasmids which direct expression of GAL4-AD fused upstream of VP16 amino-terminal 379, 369 or 335 residues, respectively. The VP16 fragments used to construct

these plasmids were obtained from pSP-T379, pSP-T369 and pSP-T335 which contain the corresponding carboxy-terminal truncated mutant of VP16, generated by subcloning previously described Bam HI insertion mutants of VP16 (Werstuck and Capone, 1989a) between Sal I and Bam HI sites of pSPUTK. The VP16 inserts used to clone into GAL4-AD fusion vector were excised from the corresponding plasmids with Nco I (at codon 1) and Sac I (present in the MCS of pSPUTK) and ligated to the GAL4 activation domain fusion vector obtained from pCADVP16₄₀₄ as described above.

pCDBVP16₂₉₉ and pCDBVP16₂₅₀ are yeast low copy expression vectors, coding for the amino-terminal 299 or 250 (respectively) residues of VP16 fused in-frame downstream of GAL4-DBD. The construction and the maps for these plasmids are described in 4.2.1 and Fig.7.

3.2.2. Cloning internal in-frame deletion mutants of VP16 into GAL4 fusion vector

pCADVP16_{Δ141-178} (Fig.4) is a yeast low copy number vector which encodes GAL4-AD fused upstream of the 424 amino acids of VP16 with internal in-frame deletion between amino acids 141 and 178. VP16 fragment used to construct this plasmid was obtained from pSP-Δ141-178 which contains the entire coding sequence of VP16 with internal in-frame deletion between codons 141 and 178, generated by substitution of Sal I fragment of VP16 from a previously described plasmid (Werstuck and Capone, 1989a) into pSPUTK-65. VP16 insert was excised from pSP-Δ141-178 with Nco I (at codon 1) and Sac I (at codon 424) and ligated to the compatible ends of the GAL4-AD

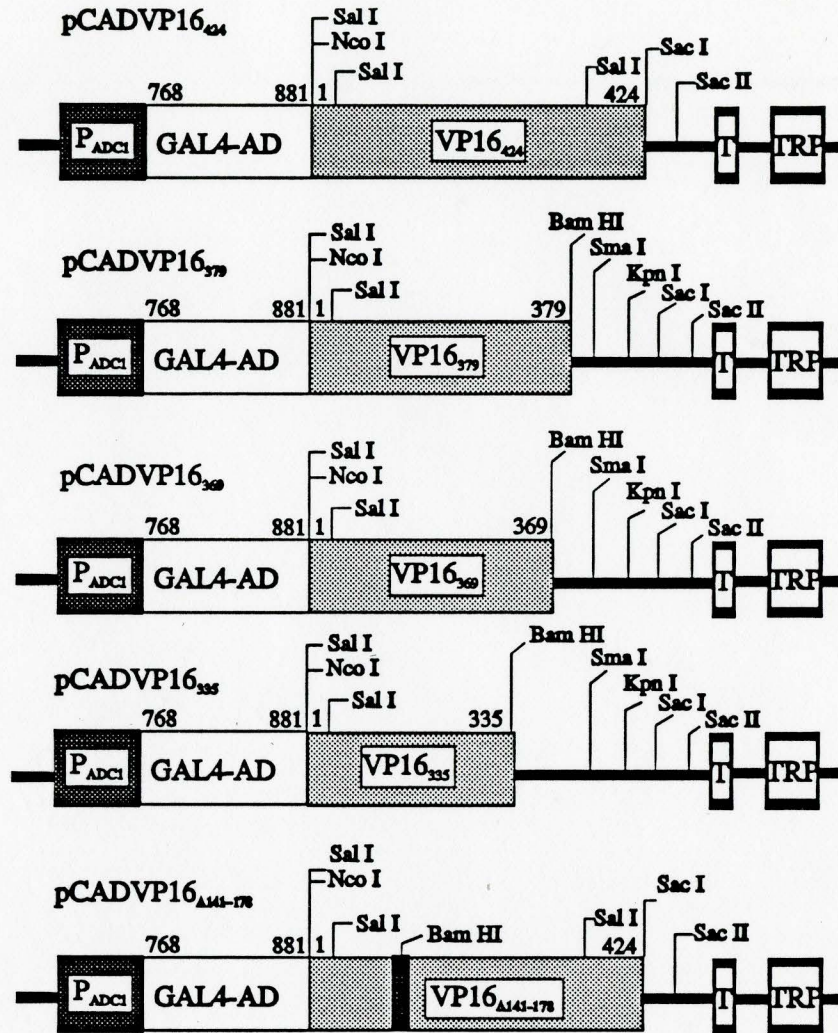


Fig. 4 pCADVP16₄₂₄, pCADVP16₃₇₉, pCADVP16₃₆₉ and pCADVP16₃₃₅ direct expression of GAL4-AD fused upstream of VP16 amino terminal 424, 379, 369 and 335 amino acids, respectively. pCADVP16_{Δ141-178} encodes for GAL4-AD fused upstream of the amino terminal 424 amino acids of VP16 with internal in-frame deletion between codons 141 and 178. All plasmids carry the promoter (P) and the terminator (T) of the yeast ADC1 gene, and TRP as selectable marker in yeast.

fusion vector obtained from pCADVP16₄₀₄ as described above (3.2.1). An additional Bam HI site is present in the VP16 ORF which had been introduced during the construction of the internal deletion (Fig.4).

pCDBVP16_{Δ25-178} is a yeast low copy plasmid which directs expression of GAL4-DBD fused upstream of the amino-terminal 424 residues of VP16 with internal in-frame deletion spanning codons 25 to 178. The construction and the map of this plasmid are shown in 4.2.2 and Fig.8.

3.2.3. Analysis of the interaction of the deletion mutants of VP16 with vhs

To test the ability of VP16 deletion mutants to interact with vhs, each of the described above plasmids expressing VP16 derivatives fused to GAL4, was introduced together with GAL4-vhs hybrid encoding plasmid into yeast PCY2 using the high efficiency LiAc transformation protocol. Transformation of yeast with GAL4-VP16 derivatives expressing plasmids alone was carried out as a control. Yeast transformants were selected on Sc media lacking the appropriate amino acids, as described previously. The interaction of the corresponding VP16 deletion mutants with vhs was monitored by measuring β -galactosidase activity of the obtained transformants. The yeast were assayed first using colony filter protocol, individual transformants were grown in selective medium for the plasmid and β -galactosidase activity was determined. The obtained results are presented in Table 3. The values for β -galactosidase activity are the averages of at least three independent transformants assayed in duplicate and normalized to cell density

Table 3

Yeast transformed with:		β -galactosidase activity	
GAL4-DBD hybrid	GAL4-AD hybrid	filters (colour)	liquid culture (U)
DBD	-	white	< 1
-	AD	white	< 1
DBD	AD	white	< 1
DBD-vhs	AD-VP16 ₄₂₄	blue	63 ± 10
DBD-vhs	AD-VP16 ₄₀₄	blue	86 ± 8
DBD-vhs	AD-VP16 ₃₇₉	blue	70 ± 7
DBD-vhs	AD-VP16 ₃₆₉	blue	77 ± 4
DBD-vhs	AD-VP16 ₃₃₅	white	< 1
DBD-VP16 ₂₉₉	AD-vhs	white	< 1
DBD-VP16 ₂₅₀	AD-vhs	white	< 1
DBD-VP16 _{Δ25-178}	AD-vhs	white	< 1
DBD-vhs	AD-VP16 _{Δ141-178}	white	< 1
-	AD-VP16 ₄₂₄	white	< 1
-	AD-VP16 ₄₀₄	white	< 1
-	AD-VP16 ₃₇₉	white	< 1
-	AD-VP16 ₃₆₉	white	< 1
-	AD-VP16 ₃₃₅	white	< 1
DBD-VP16 ₂₉₉	-	white	< 1
DBD-VP16 ₂₅₀	-	white	< 1
DBD-VP16 _{Δ25-178}	-	white	< 1
-	AD-VP16 _{Δ141-178}	white	< 1
DBD-vhs	-	white	< 1
-	AD-vhs	white	< 1

(OD₆₀₀). The standard errors are indicated.

As expected, β -galactosidase activity was not detected in any of the control transformants that expressed VP16- or vhs-hybrid proteins alone, or GAL4 DNA binding domain and/or GAL4 activation domain, showing inability of the corresponding proteins to activate transcription of the reporter lacZ gene.

No β -galactosidase activity was detected in yeast co-expressing vhs and VP16 deletion mutants encompassing the first 250, 299 or 335 amino acids, respectively. The same result was obtained with yeast co-expressing vhs and VP16 internal in-frame deletion mutants spanning region between amino acids 25-178 and 141-178, respectively. The absence of β -galactosidase activity indicated the inability of the above mentioned VP16 derivatives to interact with vhs.

β -galactosidase activity was detected in yeast co-expressing VP16 deletion mutants encompassing the first 424, 404, 379 and 369 amino acids, respectively, showing that the deleted region was not important for interaction of VP16 with vhs which led to stimulation of transcription of the reporter lacZ gene.

3.3. Discussion

The obtained data (summarized in Fig.5) demonstrate that VP16 and vhs form a complex *in vivo* and VP16 residues required to support this interaction coincide with those identified as necessary for binding to vhs *in vitro* (Smibert *et al.*, 1993). VP16

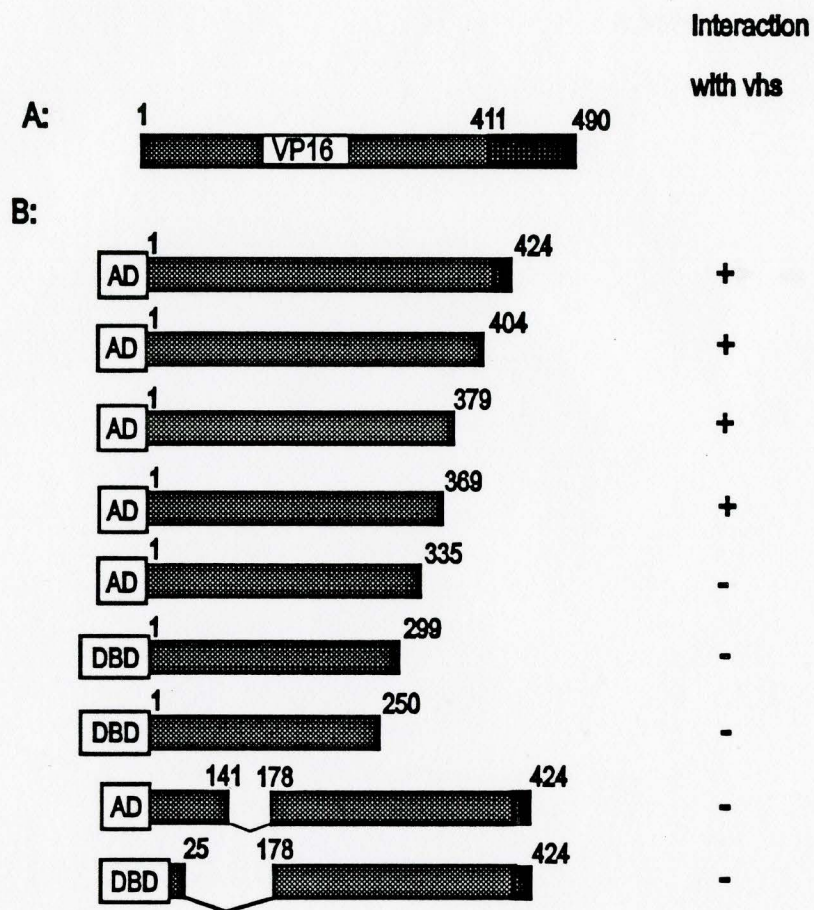


Fig. 5 VP16 interacts with vhs *in vivo*

A). Map of VP16 showing the position of carboxy-terminal activation domain (residues 411 to 490). B). Schematic diagram of GAL4-VP16 derivatives (numbers correspond to VP16 codons). DBD and AD represent GAL4 DNA binding and activation domains, respectively.

sequence contained within the first 369 amino acids is sufficient for interaction with vhs. Any larger carboxy-terminal truncation completely abolished VP16 binding to vhs suggesting that the region between residues 335 and 369 is involved in protein-protein interaction with vhs. The inability of VP16 derivatives with small internal deletions to form a complex with vhs suggests that improper conformation of the protein might be the reason for the eliminated interaction. Recent data (discussed in details in section II) indicates that the region encompassing residues 360-390 is important for direct interaction of VP16 with Oct-1 and VCAF-1 (Stern and Herr, 1991; Hayes and O'Hare, 1993) and vhs is able to block the formation of VP16 induced complex on TAATGARAT element when present at high concentration (Smibert *et al.*, 1993). Thus, it is possible that the binding site for vhs overlaps with the binding sites for the cellular factors.

Considering the dual role of VP16 and vhs as structural components of the viral particle and regulatory factors involved in transactivation or protein synthesis shutoff, respectively, several possibilities arise to explain the functional significance of their interaction for HSV-1 infection.

As both VP16 and vhs are located in the virion tegument, it is possible that VP16-vhs complex has an important structural role in the assembly of the virion. The fact that VP16 is essential for virion assembly (Ace *et al.*, 1988; Weinheimer *et al.*, 1992), while vhs is dispensable (Smibert and Smiley, 1990; Fenwick and Everett, 1990), suggests that VP16 might be involved in packaging vhs in the tegument rather than *vice versa*.

Alternatively, VP16-vhs interaction might be important for modulating their

function as transactivator (if vhs regulates VP16) or as factor involved in protein synthesis shutoff (if VP16 regulates vhs) during infection. As VP16 is much more abundant than vhs in both virions and the infected cells it is unlikely that vhs is able to modulate the activity of VP16. It is interesting to speculate that VP16 interaction with vhs late during infection might block vhs function and protect viral mRNAs from degradation, by directing the newly synthesized vhs protein to the virion assembly pathway.

The study of VP16-vhs interaction is important for understanding the function of these proteins during HSV infection and, more generally, the role of protein-protein interactions for modulation of activity of regulatory factors.

Section II

4. VP16 lacking the acidic activation domain activates transcription in yeast

The activation domain of VP16, located at the carboxy-terminus of the protein (amino acids 411-490), has been shown to function as a potent activator of transcription in mammalian and yeast cells (Sadowski *et al.* 1988; Cousens *et al.*, 1989). It belongs to the family of acidic transactivators which includes yeast GAL4 and GCN4 activators as well. VP16 deleted for this activation domain is not able to stimulate expression of a reporter gene in mammalian cells (Triezenberg *et al.*, 1988; Greaves and O'Hare, 1988). Assuming that the mechanism of transactivation by VP16 is conserved in mammalian and yeast cells, we expected that VP16 devoid of its activation domain would not act as transcriptional activator when fused to DNA binding domain in yeast.

4.1. VP16 (amino acids 1-404) fused to GAL4-DBD is a functional transcriptional activator in yeast

Surprisingly, the expression of the first 404 amino acids of VP16 fused to GAL4-DBD activated transcription of the regulated by GAL4-responsive promoter, lacZ reporter gene in yeast, even in the absence of the AAD (see 3.1.1. and Table 1). The increased level of β -galactosidase activity (142 ± 6 U) was clearly caused by VP16₄₀₄ hybrid protein as the activity was not detectable in yeast expressing GAL4 DNA binding

domain alone. This result demonstrated that VP16 lacking the acidic activation domain, functions as a strong transcriptional activator in yeast when provided with DNA binding domain.

As VP16 caused transactivation was easily monitored by assaying β -galactosidase activity, the system could be exploited to map the functional domain of VP16 involved in stimulation of transcription in yeast. In order to identify the region of VP16 sufficient for activation function in yeast, a number of VP16 deletion mutants were fused downstream of GAL4-DBD and their ability to enhance expression of the reporter lacZ gene was tested in yeast.

4.2. Mapping the region of VP16 involved in transactivation in yeast

4.2.1. Cloning carboxy-terminal deletion mutants of VP16 into GAL4-DBD fusion vector

pCDBVP16₄₂₄ (Fig.6a) is a yeast low copy vector, which directs expression of the amino-terminal 424 residues of VP16 fused downstream of GAL4-DBD. The coding sequence for VP16 used to construct this plasmid, was excised from pSPUTK-65 (contains the VP16 entire ORF) with Nco I (at codon 1) and Sac I (at codon 424). The VP16₄₂₄ insert was then ligated to GAL4-DBD fusion vector, obtained from pCDBVP16₄₀₄ (Fig.1) after first cutting out the VP16 sequence with Nco I (at codon 1) and Sac I (in the MCS of the plasmid).

pMAVP16₄₁₁ (Fig.6b) is a yeast high copy expression vector, encoding for GAL4-DBD fused upstream of the amino-terminal 411 residues of VP16. The VP16₄₁₁ coding sequence used to construct this plasmid was excised from pSPUTK-65 with Sal I (present at codons 1 and 411) and then cloned into Sal I site in the MCS of the high copy GAL4-DBD fusion vector pMA424 (see Materials and Methods). The correct reading frame for VP16₄₁₁ was obtained by linearizing the generated plasmid at the unique Eco RI site (present at the fusion point between GAL4-DBD and VP16 fragment in the MCS), blunting with mung bean nuclease and re-ligating the plasmid. The correct reading frame for VP16₄₁₁ fused downstream of GAL4-DBD was confirmed by DNA sequencing at the fusion point.

pCDBVP16₃₇₉, pCDBVP16₃₆₉, pCDBVP16₃₃₅, pCDBVP16₂₉₉ and pCDBVP16₂₅₀ (Fig.7) are yeast low copy expression vectors, encoding GAL4 DNA binding domain fused upstream of VP16 ORF for the amino-terminal 379, 369, 335 299 and 250 residues, respectively. The VP16 coding sequence used to construct these hybrid proteins, was excised with Nco I (at codon 1) and Sac I (in the MCS) from pSP-T379, pSP-T369, pSP-T335, pSP-T299 and pSP-T250, respectively, which contain the corresponding truncated mutant of VP16 between Sal I and Bam HI sites of pSPUTK, subcloned from previously described Bam HI insertion mutants of VP16 (Werstuck and Capone, 1989a). These carboxy-terminal derivatives of VP16 were ligated to the compatible ends of the GAL4-DBD fusion vector, obtained from pCDBVP16₄₀₄ (Fig.1) as described above.

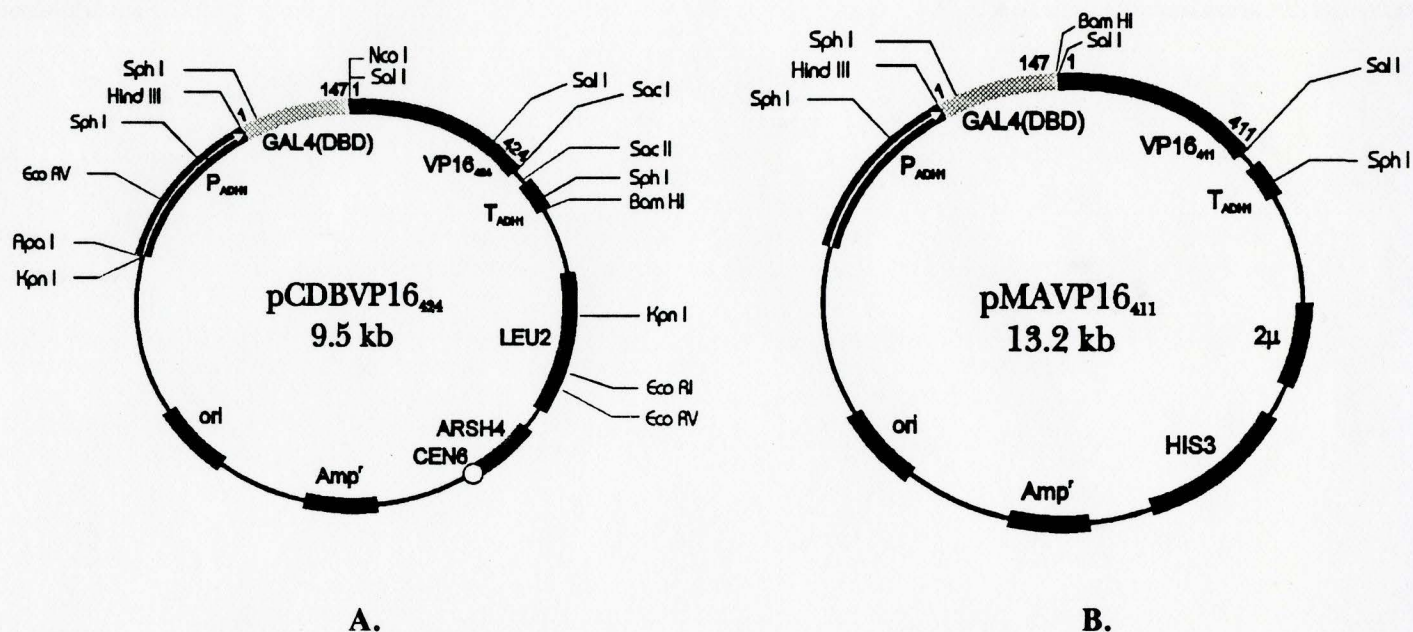


Fig. 6 A). pCDBVP16₄₂₄ is a yeast low copy vector, directing expression of the amino terminal 424 residues of VP16 fused downstream of GAL4-DBD. It carries LEU2 as selectable marker in yeast and ARS/CEN sequence for stable replication and segregation in yeast. B). pMAVP16₄₁₁ is yeast high copy expression vector coding for the amino terminal 411 residues of VP16 fused downstream of GAL4-DBD. It has HIS3 as selectable marker in yeast and 2μ sequence for maintaining high copy number of the plasmid in yeast. Both hybrid proteins are expressed under control of the constitutive promoter for yeast ADC1 gene. (Adapted from Chevray and Nathans, 1992).

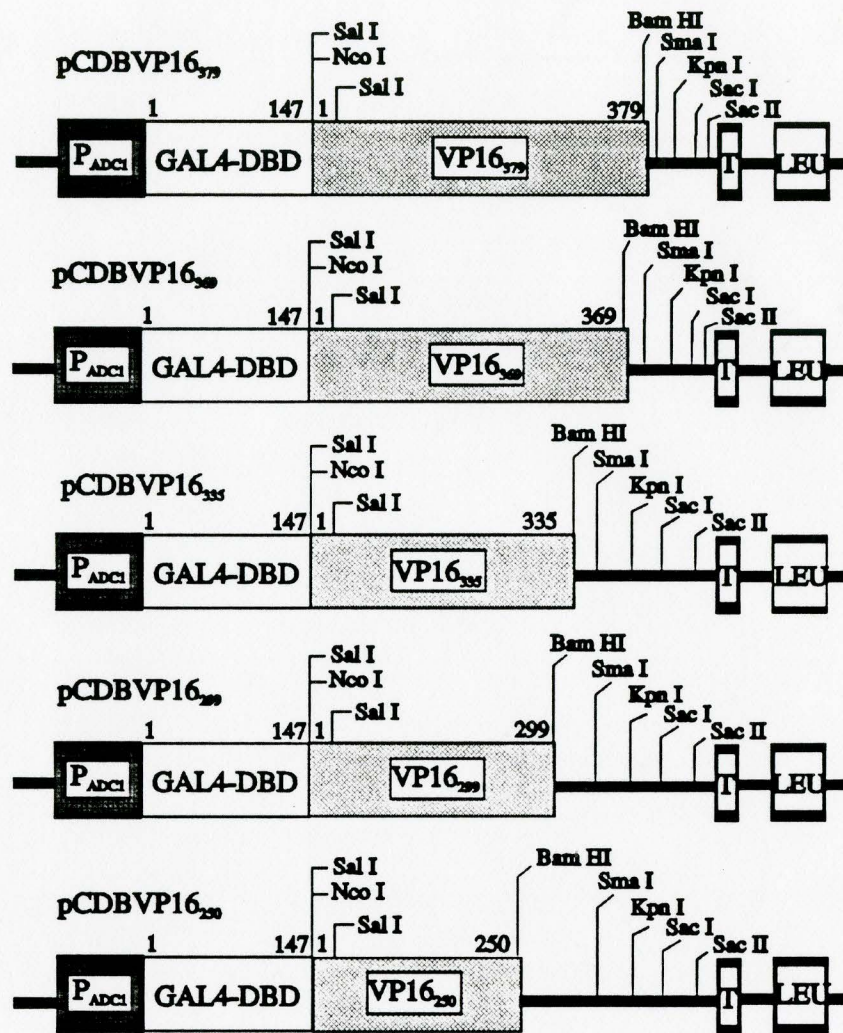


Fig. 7 pCDBVP16₃₇₉, pCDBVP16₃₆₉, pCDBVP16₃₃₅, pCDBVP16₂₉₉ and pCDBVP16₂₅₀ direct expression of GAL4-DBD fused upstream of VP16 amino terminal 379, 369, 335, 299 and 250 residues, respectively. All plasmids carry the promoter (P) and the terminator (T) of the yeast ADC1 gene, and LEU as selectable marker in yeast.

4.2.2. Cloning internal in-frame deletion mutants of VP16 into GAL4-DBD fusion vector

pCDBVP16_{Δ25-178} and pCDBVP16_{Δ141-178} (Fig.8a) are yeast low copy expression vectors, coding for GAL4-DBD fused upstream of the amino-terminal 424 residues of VP16 with an internal in-frame deletion spanning codons 25 to 178 or 141 to 178, respectively. The VP16 coding sequence used to construct these plasmids was obtained from pSP-Δ25-178 and pSP-Δ141-178 which contain VP16 ORF with the corresponding internal deletion, generated by replacement into pSPUTK-65 the respective Sal I fragment of VP16 derivative from previously described plasmids (Werstuck and Capone, 1989a). VP16_{Δ25-178} and VP16_{Δ141-178} inserts were cut out with Nco I (at codon 1) and Sac I (at codon 424) and ligated to the compatible ends of GAL4-DBD fusion vector obtained from pCDBVP16₄₀₄ (Fig.1) as described earlier.

4.2.3. Cloning the acidic activation domain of VP16 into GAL4-DBD fusion vector

The activation domain of VP16 strongly stimulates transcription in yeast, when fused to GAL4 DNA binding domain (Cousens *et al.*, 1989). In order to compare the transactivation ability of the described above VP16 deletion mutants to that of its acidic activation domain, the carboxy-terminal 79 amino acids of VP16 were cloned into GAL4-DBD fusion vector. pCDBVP16_{ΔAD} (Fig.8b) is a yeast low copy vector, directing expression of the acidic activation domain of VP16 (amino acids 411 to 490) fused downstream of GAL4-DBD. It was generated by subcloning the Sal I (present at codon

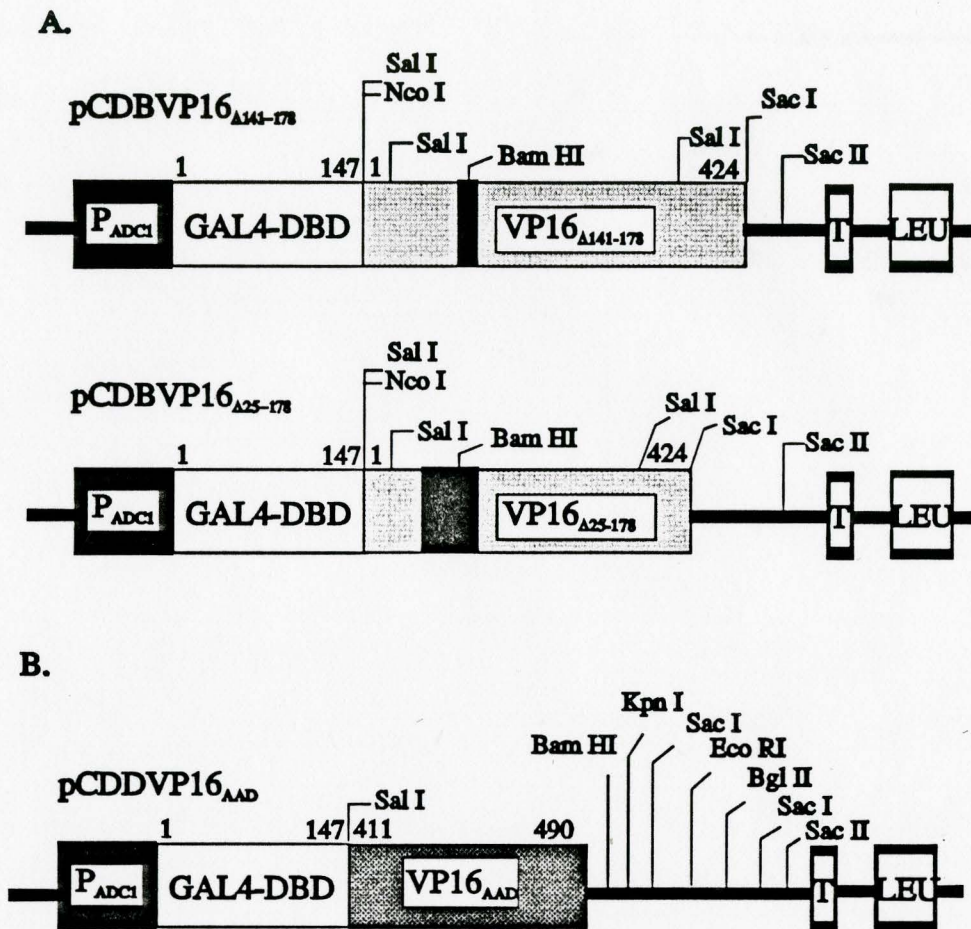


Fig.8 A). pCDBVP16 $_{\Delta 141-178}$ and pCDBVP16 $_{\Delta 25-178}$ are yeast low copy expression vectors, coding for GAL4-DBD fused upstream of the amino-terminal 424 residues of VP16 with an internal in-frame deletion spanning codons 141 to 178 or 25 to 178, respectively. B). pCDBVP16 $_{AAD}$ is a similar yeast low copy vector, directing expression of the acidic activation domain of VP16 (residues 411 to 490) fused downstream of GAL4-DBD. All plasmids have the promoter (P) and terminator (T) of ADC1 gene and LEU as selectable marker in yeast.

411) to Bgl II (present in the MCS) fragment of pSPUTK-65 into the corresponding sites in the MCS of GAL4-DBD fusion vector pPC97.

4.2.4. Analysis of transactivation function of VP16 deletion mutants in yeast

The VP16 derivatives, fused in-frame to GAL4 DNA binding domain, were introduced into yeast PCY2 by LiAc transformation protocol and Leu⁺ transformants were selected. The ability of the VP16 deletion mutants to activate transcription was determined by measuring β -galactosidase activity directly from plates (colony filter assay) and in liquid cultures of individual transformants grown in selective medium for the plasmid. The level of transactivation exhibited by each of VP16 derivatives tested is shown in Table 4. The results (summarized in Fig.9) demonstrated that VP16 missing carboxy-terminal residues up to amino acid 369 was still able to stimulate transcription of the reporter gene in yeast. The level of transactivation was 25 to 45% of that obtained with GAL4-VP16_{AAD}, a potent yeast and mammalian transactivator (Sadowski *et al.*, 1988). Thus, the carboxy-terminal 120 amino acids were dispensable for the activation function of VP16 in yeast. However, transactivation of VP16 deletion mutants was dependent on DNA binding as the same VP16 derivatives cloned into GAL4 activation domain fusion vector were not able to stimulate transcription of the reporter gene (see 3.2.3 and Table 3). Carboxy-terminal deletions beyond codon 369 as well as the internal in-frame deletions of VP16 rendered the hybrid protein completely inactive as judged by

Table 4:

Yeast transformed with:	β -galactosidase activity	
GAL4-DBD hybrid	Filters (colour)	Liquid culture (U)
DBD	white	< 1
DBD-VP16 _{AAD}	blue	312 \pm 2
DBD-VP16 ₄₂₄	blue	74 \pm 2
DBD-VP16 ₄₁₁ *	blue	2 \pm 0.5
DBD-VP16 ₄₀₄	blue	142 \pm 9
DBD-VP16 ₃₇₉	blue	72 \pm 5
DBD-VP16 ₃₆₉	blue	101 \pm 7
DBD-VP16 ₃₃₅	white	< 1
DBD-VP16 ₂₉₉	white	< 1
DBD-VP16 ₂₅₀	white	< 1
DBD-VP16 _{Δ25-178}	white	< 1
DBD-VP16 _{Δ141-178}	white	< 1

The presented values of β -galactosidase activity are the averages of three to five independent transformants assayed in duplicate and normalized to cell density (OD₆₀₀).

The standard errors are indicated. (*) DBD-VP16₄₁₁, unlike the rest of the hybrid proteins, is expressed from a high copy number plasmid.

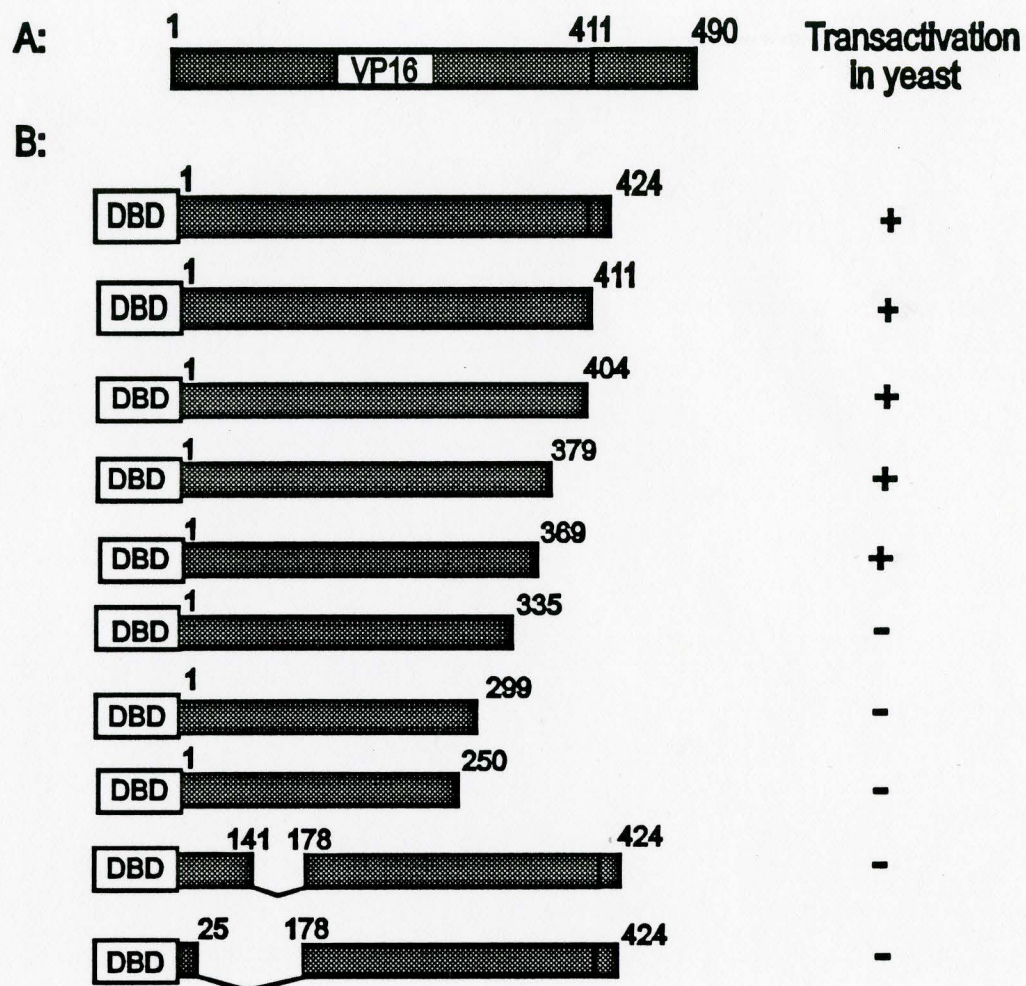


Fig. 9 VP16 lacking the AAD activates transcription in yeast.

A). Map of VP16 showing the position of carboxy-terminal activation domain (residues 411 to 490). B). Schematic diagram of GAL4-VP16 derivatives (numbers correspond to VP16 codons). DBD represents GAL4 DNA binding domain (amino acids 1 to 147).

the absence of β -galactosidase activity (Table 4). Thus, transactivation in yeast requires a large domain within the amino terminal 369 amino acids of VP16.

4.2.5. Analysis of protein expression levels of VP16 hybrids in yeast

The inability of VP16 internal or truncated beyond codon 369 deletion mutants to stimulate transcription could be due to inadequate expression or instability of the corresponding VP16 derivatives in yeast rather than a deficiency in transactivation function. To determine the level of expression of the various VP16 fusion proteins, Western blot analysis using anti-GAL4 antiserum was performed.

Extracts were prepared from yeast harbouring the different GAL4-VP16 derivatives using the glass bead disruption method and equivalent amounts of each sample were fractionated by SDS PAGE. After transfer of the proteins to nitrocellulose, the filters were incubated with rabbit anti-GAL4 polyclonal antibody specific for the GAL4 DNA binding domain. HRP-linked donkey anti-rabbit IgG was used as a secondary antibody and the complexes were detected by enhanced chemiluminescence using the ECL Western blotting detection system. The expected size VP16 fusion proteins were readily detected, as shown in Fig.10. GAL4-VP16₃₃₅ was barely detectable (lane f) and GAL4-VP16₂₉₉ was obscured by its co-migration with a cross-reacting yeast protein (lane g). The steady-state levels of GAL4-VP16₄₂₄ and GAL4-VP16₄₀₄ were comparable to GAL4-VP16_{AAD} (lanes a, c and k). This analysis showed that the loss of transactivation function of some of GAL4-VP16 derivatives, such as GAL4-VP16 _{Δ 141-178} and GAL4-

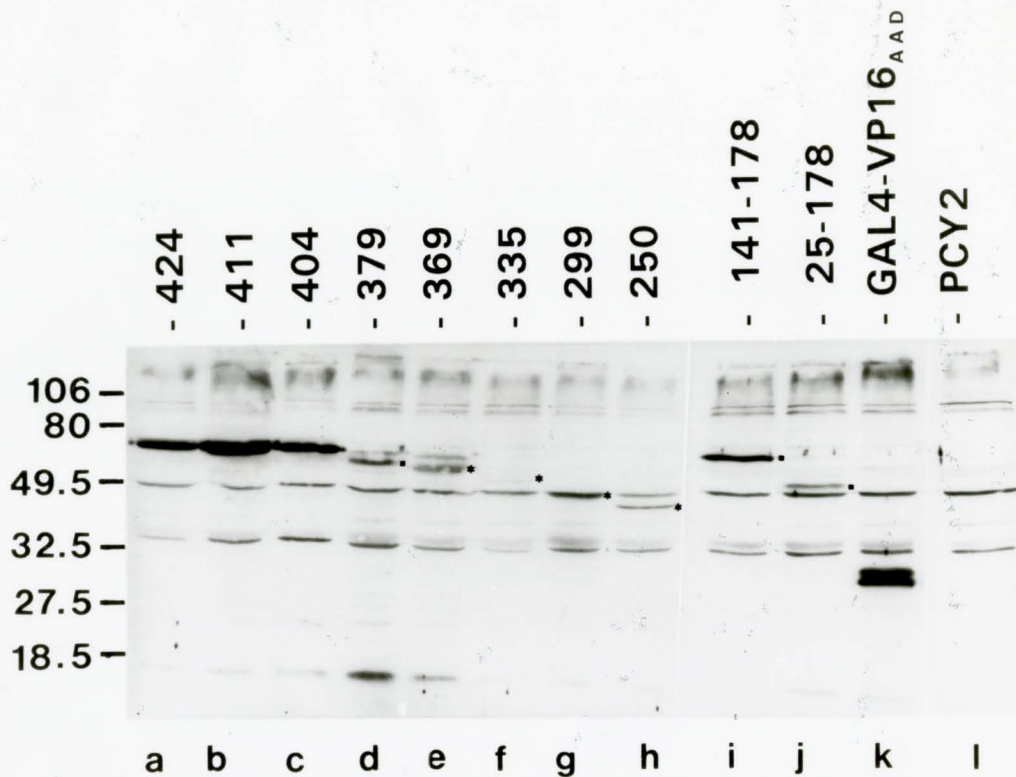


Fig 10 Expression of GAL4-VP16 fusion proteins

The various GAL4-VP16 derivatives expressed in yeast are indicated at the top of the figure (the numbers correspond to VP16 codons). VP16_{AAD} represents the acidic activation domain of VP16 fused to GAL4. PCY2 is a control using untransformed yeast. The position of some of the fusion proteins is highlighted with an asterisk.

VP16_{Δ25-178}, in yeast could not be attributed to inadequate expression level. The results also showed that the very weak transactivation exhibited by GAL4-VP16₄₁₁ hybrid (see Table 4) was not due to the low expression level of this protein, as it was present in much higher concentration than GAL4-VP16₃₆₉, which caused substantially stronger transactivation of the reporter gene. VP16₄₁₁, unlike the rest of VP16 derivatives, was expressed from high copy number plasmid, thus the reduction in activation could be due to VP16 mediated squelching, which has been shown to occur when transactivators are present in high concentration (see Discussion).

4.3. VP16 lacking the acidic activation domain does not activate transcription in mammalian cells even if provided with a DNA binding domain

The above findings were unexpected as VP16 lacking the acidic activation domain is completely inactive in transactivation in mammalian cells (Triezenberg *et al.*, 1988; Greaves and O'Hare, 1989; Werstuck and Capone, 1989a). Although the presence of an additional upstream activation domain in VP16 that functions only in yeast could not be excluded, it was possible that fusion to DNA binding domain induced a conformational change in VP16 and exposed an otherwise concealed activation domain. We therefore determined whether VP16 missing the AAD could activate transcription in mammalian cells if it was directly tethered onto a responsive promoter. The same VP16 derivatives were cloned into mammalian GAL4-DBD fusion vector and their ability to transactivate

expression of regulated by GAL4-responsive promoter CAT reporter gene was assayed in mammalian cells.

4.3.1. Cloning C-terminal deletion mutants of VP16 into GAL4-DBD mammalian fusion vector

pSGVP16₄₀₄, pSGVP16₃₇₉, pSGVP16₃₆₉ and pSGVP16₃₃₅ (Fig. 11) are mammalian vectors expressing GAL4-DBD fused upstream of VP16 amino-terminal 404, 379, 369 or 335 residues, respectively. The corresponding coding sequence for these carboxy-terminal deletion mutants of VP16 was excised from pCDBVP16₄₀₄, pCDBVP16₃₇₉, pCDBVP16₃₆₉ and pCDBVP16₃₃₅ described earlier. VP16 containing plasmids were linearized with Nco I (at codon 1), blunted with Klenow polymerase and cut with Sac I (present in the MCS). Thus obtained VP16 inserts were cloned between Sma I and Sac I sites in the MCS of GAL4-DBD fusion vector pSG424 (see Materials and Methods).

4.3.2. Analysis of transactivation function of VP16 fusion proteins in mammalian cells

The ability of VP16 deletion mutants, provided with DNA binding domain, to function as transcriptional activators in mammalian cells was tested by co-transfection cells with plasmid expressing VP16 derivative fused to GAL4-DBD and CAT reporter gene containing upstream five GAL4 binding sites (pGAL4₅/E1bCAT). The level of CAT activity in the transfected cells is indicative for the ability of VP16 mutants to stimulate

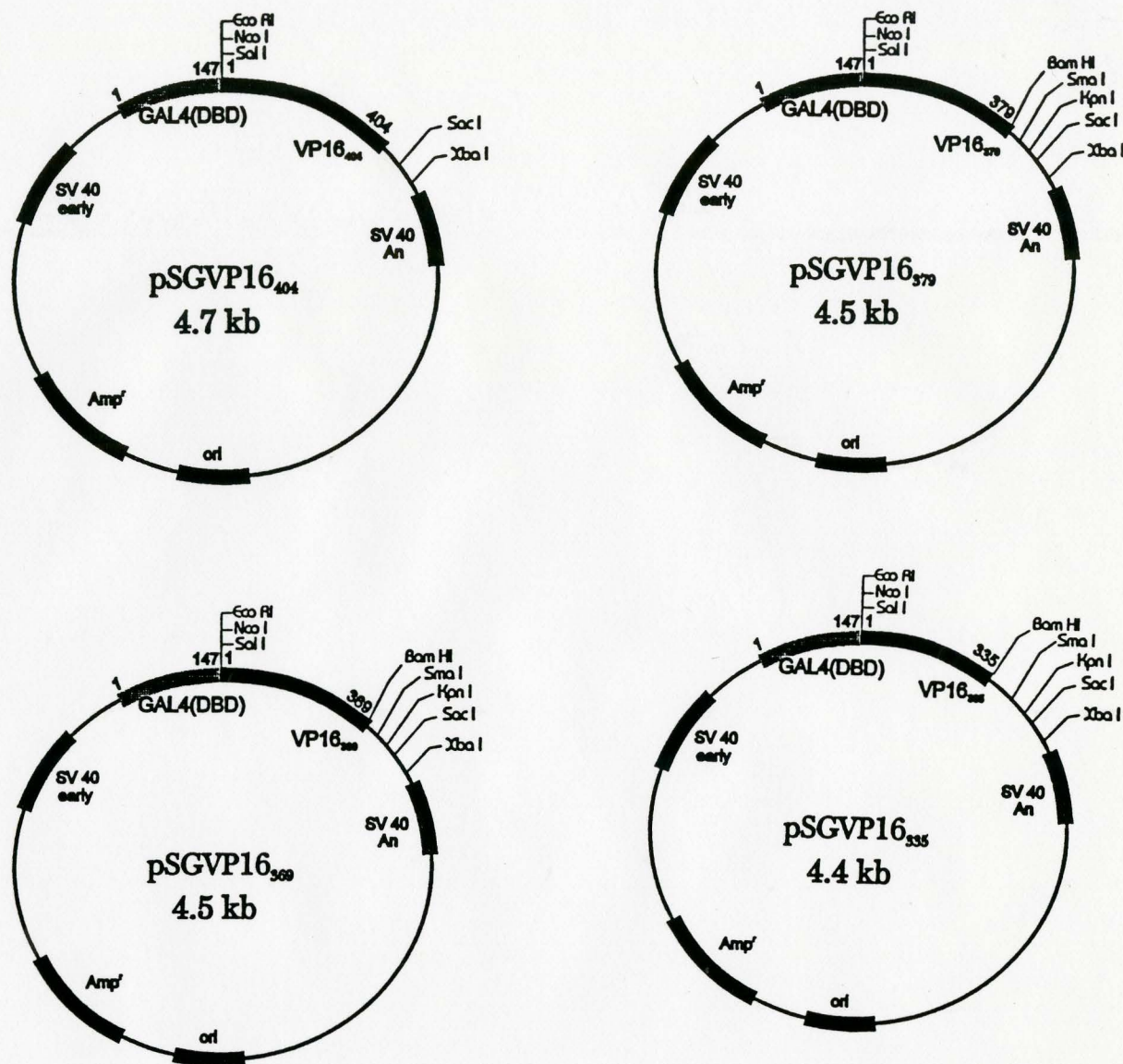


Fig.11 pSGVP16₄₀₄, pSGVP16₃₇₉, pSGVP16₃₆₉ and pSGVP16₃₃₅ are mammalian vectors expressing VP16 amino-terminal 404, 379, 369 or 335 residues, respectively, fused downstream of GAL4-DBD (the numbers correspond to VP16 and GAL4 codons). The plasmids contain also the SV40 ori/early promoter region and a polyA signal.

expression of the reporter gene. COS-1 cells were transfected with increasing amounts of the described above pSGVP16 plasmids along with the CAT reporter gene, using the calcium phosphate/DNA precipitation method. Transfection with plasmids expressing GAL4 DNA binding domain GAL4(1-147) or VP16 acidic activation domain fused to GAL4-DBD (GAL4-VP16_{AAD}), were carried out as a negative and positive control, respectively. Cells extracts were prepared 48 h post-transfection and CAT activity was measured to determine the transactivation ability of the VP16 mutants. The obtained results are shown in Fig.12. As expected, GAL4(DBD)-VP16_{AAD} strongly stimulated the expression of the reporter gene even at the lowest plasmid concentration used (lanes d-f), while GAL4 DNA binding domain was completely inactive (lanes a-c). However, neither VP16₄₀₄ (lanes g-i), nor VP16₃₆₉ (lanes j-l), fused to GAL4-DBD increased expression of the reporter gene, as judged by the absence of CAT activity. Thus, unlike in yeast, VP16 lacking the acidic activation domain does not function as a transcriptional activator in mammalian cells even if it is directly bound to a promoter.

5. Active GAL4(DBD)-VP16 fusion proteins promote cell aggregation in yeast

5.1. Phenotype of yeast transformed with different VP16 fusion proteins

An interesting phenomenon was observed when individual yeast transformants harbouring the various GAL4(DBD)-VP16 expression plasmids, were grown in liquid cultures. Wild type yeast (PCY2), as well as yeast expressing GAL4 DNA binding

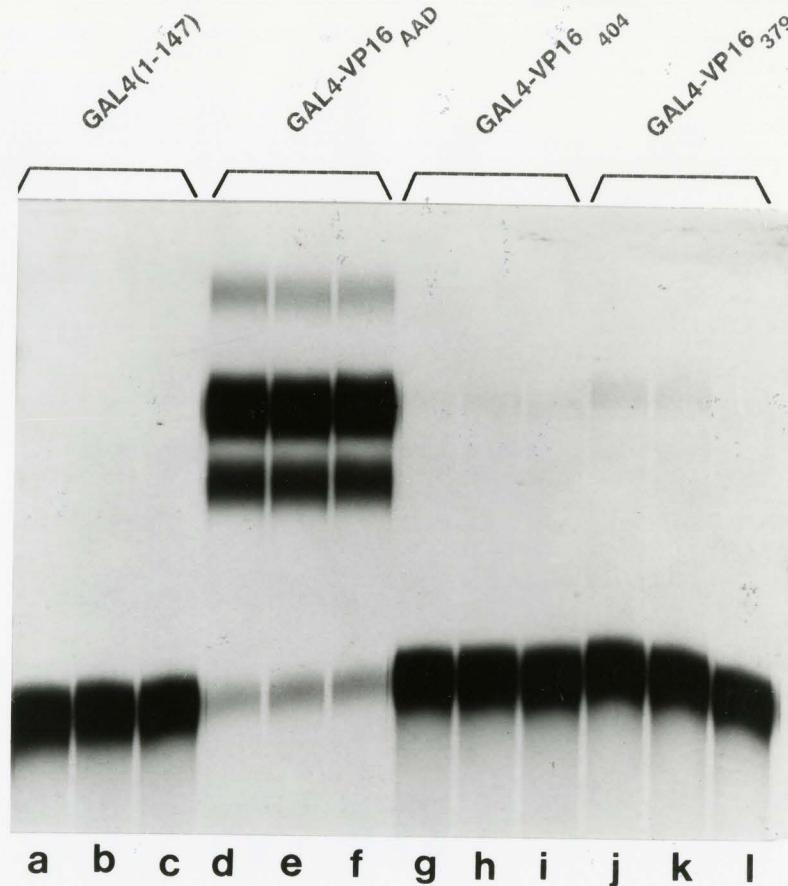


Fig.12 AAD-deleted VP16 derivatives do not function in mammalian cells when tethered directly to a promoter. CAT activity of COS-1 cells co-transfected with CAT reporter plasmid and 0.5 μ g (lanes a, d, g and j), 1 μ g (lanes b, e, h and k), or 5 μ g (lanes c, f, i and l) of plasmids expressing GAL4(1-147), GAL4-VP16_{AAD}, GAL4-VP16₄₀₄ or GAL4-VP16₃₆₉ fusion proteins.

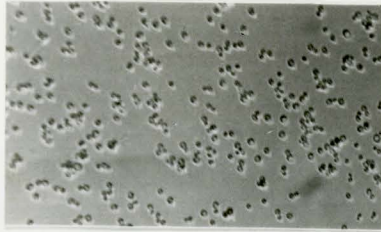
domain or inactive VP16 fusion derivatives formed a single cell suspension during the exponential growth phase. Upon approaching saturation the cells tended to aggregate into small clumps which were easily dispersed by shaking or vortexing the cultures at low speed. On the other hand, an extensive aggregation was observed with yeast transformed with the active VP16 fusion proteins even during the exponential growth (Fig.13). The aggregation phenotype was most evident in yeast expressing GAL4-VP16₃₇₉ and GAL4-VP16₃₆₉ (Fig.14) which did not form a suspension at all but aggregated into large clumps that could not be mechanically dispersed (even by vigorous vortexing) into single cells. The aggregation was dependent on DNA binding as yeast expressing VP16 derivatives fused to GAL4 activation domain did not differ from the wt phenotype. It is unlikely that accumulation of a foreign protein causing a non-specific physiological effect was responsible for aggregation as this phenotype was observed only in yeast expressing transactivation competent VP16 derivatives. However, transactivation itself was, probably, not responsible for the aggregation phenotype as yeast expressing GAL4-VP16_{AAD} under the same conditions did not aggregate (Fig.13).

5.2. Ca^{2+} dependency of the aggregation phenotype

Cell aggregation in *S. cerevisiae* is important but poorly understood phenomenon that occurs during mating (sexual agglutination) or asexually via interaction between cell surface lectins and sugar residues intrinsic to the mannan which comprises cell walls (cell flocculation) (Stratford, 1992). A distinctive characteristic for these two processes is the

A:

a: PCY2



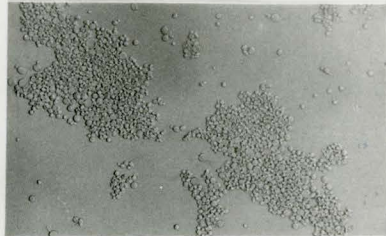
b: GAL4-VP16_{AAD}



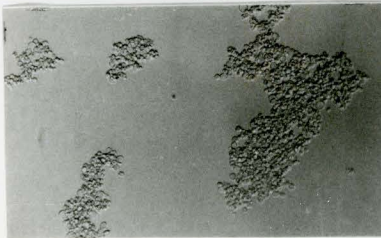
c: GAL4-VP16₄₂₄



d: GAL4-VP16₄₀₄



e: GAL4-VP16₃₇₉



f: GAL4-VP16₃₆₉



g: GAL4-VP16₂₉₉



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Fig. 13 Expression of active GAL4-VP16 derivatives promotes aggregation in yeast.

Light micrographs of *S. cerevisiae* PCY2 a) non-transformed, or transformed with plasmids expressing b) GAL4-VP16_{AAD}; c) GAL4-VP16₄₂₄; d) GAL4-VP16₄₀₄; e) GAL4-VP16₃₇₉; f) GAL4-VP16₃₆₉ or g) GAL4-VP16₂₉₉, respectively. Yeast were grown in Sc-Leu medium to mid-log phase and micrographs were taken using Nomarski optics.

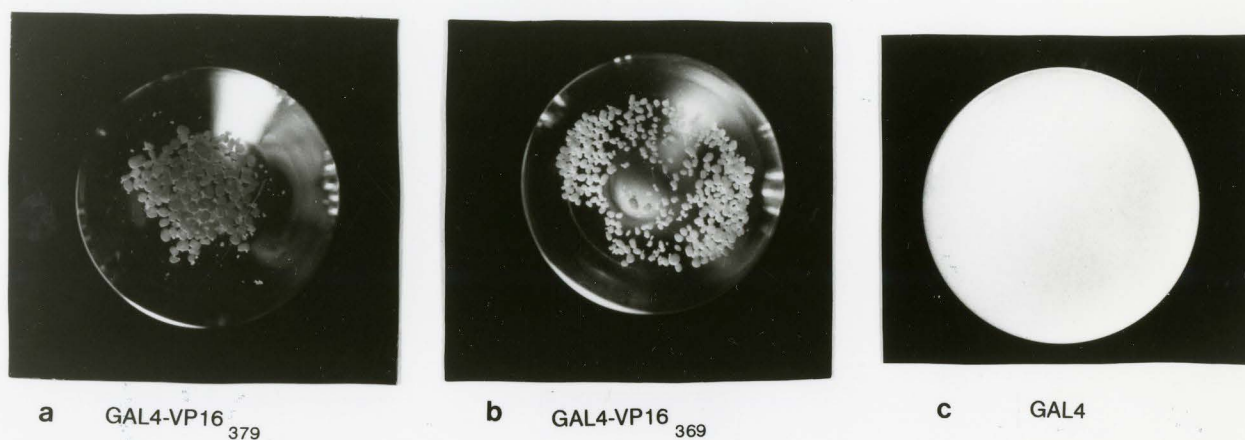


Fig. 14 Aggregation phenotype of yeast caused by active GAL4-VP16 derivatives.

Yeast transformants expressing a) GAL4-VP16₃₇₉; b) GAL4-VP16₃₆₉ or c) GAL4 DNA binding domain were grown to saturation in SC-Leu medium with constant agitation. The photographs show the bottom of the flasks used to grow the cultures.

Ca^{2+} dependence of cell flocculation while sexual agglutination is Ca^{2+} independent. Thus, sexual agglutination is not affected by the presence or absence of Ca^{2+} , while depleting the medium of Ca^{2+} leads to dispersion of the flocculated clumps into suspension. Addition of Ca^{2+} back to the depleted medium results in a rapid formation of the aggregates again.

To test if the aggregation phenotype caused by VP16 fusion proteins resembles cell flocculation or sexual agglutination, the dependence on Ca^{2+} for the formation of the aggregates was assayed. Yeast expressing DBD-VP16₃₇₉, DBD-VP16₃₆₉ and GAL4 DBD were grown in Sc-Leu medium to saturation and the following experiment was performed:

Treatment	GAL4-VP16 ₃₇₉	GAL4-VP16 ₃₆₉	GAL4-DBD
Liquid cultures	clumps	clumps	suspension
+ EDTA (20 mM)	suspension	suspension	suspension
+ Ca^{2+} (10 mM above EDTA)	clumps	clumps	suspension

Thus, the aggregation phenotype caused by the active VP16 hybrid proteins, showed characteristics typical for cell flocculation.

6. Discussion

The results presented in this section demonstrate that truncated VP16 appended onto a heterologous DNA binding domain is a transcriptional activator in yeast despite the absence of its AAD. Contrary to previous studies which have shown that VP16 deleted beyond amino acid 450 is completely inactive in mammalian cells (Triezenberg *et al.*, 1988; Greaves and O'Hare, 1989), the amino terminal 369 residues are necessary and sufficient for transactivation function in yeast. The fact that a large region of VP16 is required for activity in yeast, suggests that the conformation of the truncated polypeptide is important for transactivation. As truncated GAL4-VP16 derivatives are inactive in mammalian cells, fusion to DNA binding domain, potentially resulting in an exposure of a cryptic VP16 activation domain, can not be attributed to the observed transactivation.

One explanation for these findings is that VP16 interacts with a yeast factor that supplies an activation domain in *trans*. Consistent with this, the region required for transactivation function in yeast overlaps with the sequences of VP16 implicated in protein-protein interactions with the mammalian factors resulting in VIC assembly on TAATGARAT element. Deletion, linker insertion and point mutagenesis have identified two distinct regions of VP16 required for the protein/DNA complex formation *in vitro* and subsequent transactivation *in vivo* (Fig.15). Region 1, spanning residues 145-250, is thought to be involved in the weak intrinsic DNA binding activity of VP16 (Stern and Herr, 1991). Several studies indicate the importance of region 2 (residues 335-390), for

VIC formation through protein-protein interactions with the cellular factors. Thus, a 4 amino acid insertion at residue 369 or 379, or point mutations in this region completely eliminate transactivation function of VP16 despite the integrity of the acidic activation domain (Ace *et al.*, 1988; Werstuck and Capone, 1989a; Greaves and O'Hare, 1989; Greaves and O'Hare, 1990). Further studies have shown that the insertion at residue 379 specifically disrupts VP16 interaction with Oct-1 (Stern and Herr, 1991), while region upstream amino acid 379 is involved in binding to VCAF-1 (Popova *et al.*, 1993). Consistent with this, the specific inhibition of VIC assembly with a small polypeptide corresponding to residues 360 to 367 of VP16 (Hayes and O'Hare, 1993), is probably due to its interaction with VCAF-1. As a polypeptide corresponding to amino acids 360-393 forms a complex with DNA bound Oct-1 (Stern and Herr, 1991), the residues between 379 and 393 are most likely involved in direct interaction with Oct-1. Therefore the abolished transactivation ability of VP16 mutants in region 2 is due to their inability to interact with the cellular factors and form VIC on TAATGARAT element, which is a prerequisite for the activation function. Thus, region 2 of VP16 seems to contain the necessary information for interaction with Oct-1 and VCAF-1. Furthermore, the data (presented in section I) demonstrate that the same residues are required for interaction with vhs, indicating that this region represents a domain involved in protein-protein interactions with both cellular and viral factors.

An interaction with a yeast regulatory factor involved in control of flocculation would also explain the observed phenotypic alterations in yeast expressing the active

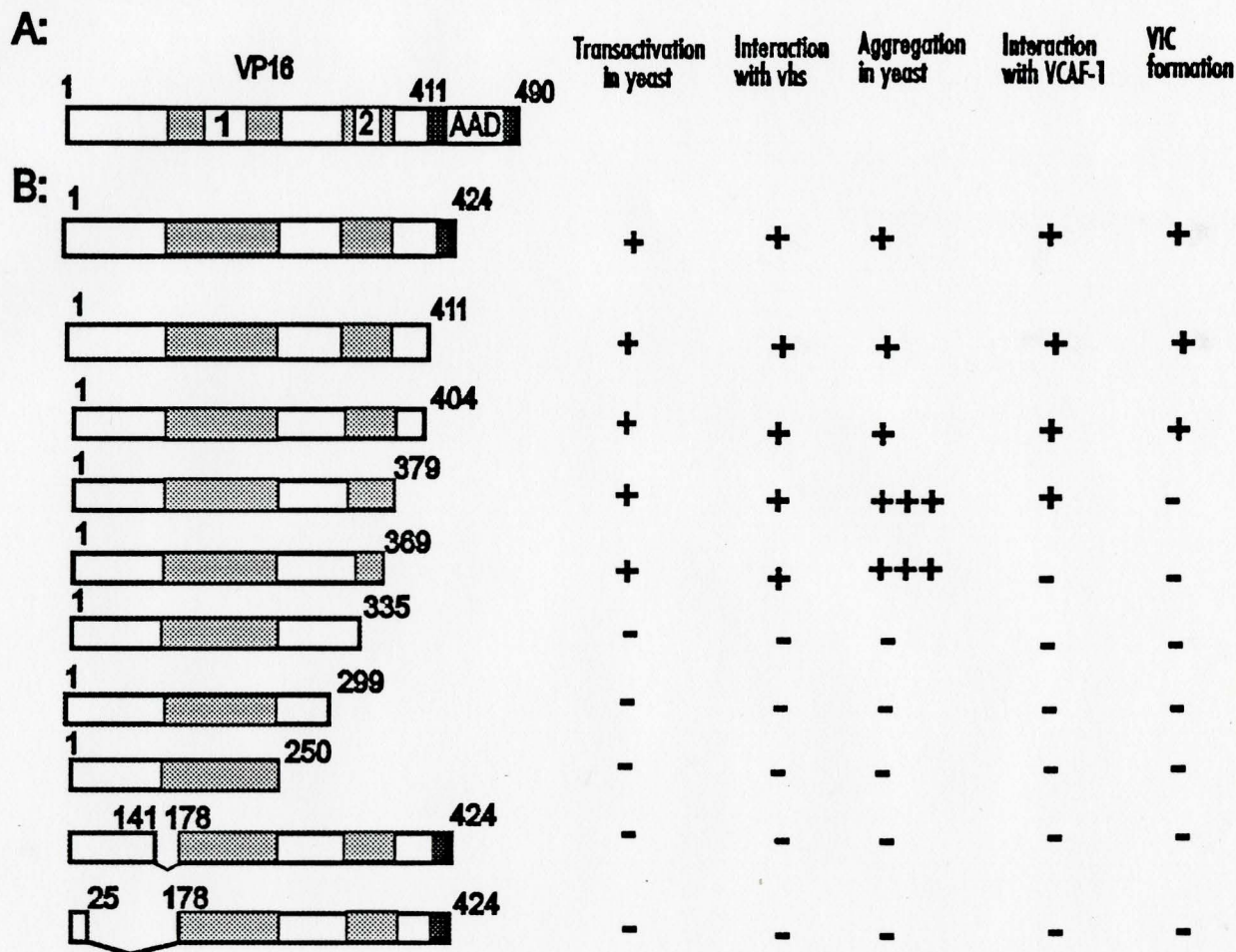


Fig. 15 Summary of mapping results derived from the present study and previous work is shown. A). Map of VP16 showing the position of the acidic activation domain (AAD). Region 1 (amino acids 141-250) and region 2 (residues 335-390) are involved in protein-protein interactions with mammalian cellular factors. B). Schematic diagram of VP16 derivatives (numbers correspond to VP16 codons).

GAL4-VP16 derivatives. Interestingly, expression of HTLV-1 transactivator Tax has been shown to cause Ca^{2+} dependent flocculation in yeast (Kramer *et al.*, 1990). The aggregation phenotype was not observed with mutant Tax deficient in transactivation in mammalian cells.

In addition, the substantially lower levels of β -galactosidase activity observed when the AAD-truncated GAL4-VP16 is expressed from high copy number plasmid compared to activity from single copy number plasmids, could be a consequence of sequestration. The reduction in activity would occur if the putative VP16-interacting yeast factor is present in limiting amounts and thus, an excess of unbound GAL4-VP16 would sequester this factor *in trans*, while the DNA bound GAL4-VP16 by itself is not able to transactivate expression of the reporter gene.

The overlapping of VP16 region involved in interaction with Oct-1, VCAF-1 and vhs (Stern and Herr, 1991; Hayes and O'Hare, 1993; Smibert *et al.*, 1993; this study) with the sequence required for transactivation/aggregation phenotype in yeast, suggests that a yeast factor(s) interacting with VP16 derivatives is responsible for the observed transactivation in yeast.

However, the alternative explanation that VP16 contains an additional yeast-specific activation domain can not be excluded. While acidic activation domains have been shown to function both in yeast and mammalian cells (Cousens *et al.*, 1989; Sadowski and Ptashne, 1989), another class of transactivators containing Glu rich activation domain is completely inactive in yeast (Pugh and Tjian, 1990). Thus, the

opposite situation where a region that functions as transactivator in yeast but is completely inactive in mammalian cells can not be ruled out. However, other than the AAD, VP16 does not appear to contain any obvious regions that conform to the properties of the different classes of activation domains so far described (Mitchel and Tjian, 1989). Also, the fact that a large region of VP16 is required for activity in yeast, is inconsistent with the relatively small size and functionally independent nature of most activation domains.

In summary, the presented results demonstrate a novel and unexpected property of VP16 which is manifested in yeast. It will be interesting to investigate the mechanisms underlying VP16-dependent transactivation and/or phenotypic alterations in yeast since they may reveal novel attributes of VP16 that are relevant to its multifunctional role during a lytic infection.

Finally, the obtained data demonstrate the possibility to exploit the two-hybrid system for detection and characterization of protein-protein interactions *in vivo*. The minimal domains required for the interaction can be easily determined and subsequent mutational analysis would identify the critical amino acid residues. There are several advantages of using the yeast two-hybrid system as compared to the variety of assays (such as co-immunoprecipitation, affinity chromatography, chemical crosslinking) for studying protein interactions *in vitro*. These methods usually require high protein concentrations, thus raising the question about the biological relevance of the observed

interaction. In the two-hybrid system not only the necessity of high protein content is circumvented, but the proteins under study are most likely expressed in amount similar to their physiological concentration in cells. In addition, it is possible that the interaction between the two proteins in the yeast system is further stabilized by consequent interaction with the basal transcription factors thus increasing the sensitivity of the assay.

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