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By

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ABSTRACT

MUTATIONAL AND FLUORESCENCE ANALYSIS OF A TRANSCRIPTIONAL ACTIVATION DOMAIN OF THE VP16 PROTEIN OF HERPES SIMPLEX VIRUS

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VP16 is an immediate early gene activator of herpes simplex virus. The activation domain of VP16 resides in the carboxyl-terminal 80 amino acids. The VP16 activation domain can be further divided into two subdomains, VP16N (412-450) and VP16C (450-490), each of which is independently capable of activating transcription when fused to a DNA binding domain. The VP16N activation domain has been previously characterized and the most critical amino acid residues for transcriptional activation have been identified as aromatic Phe 442, and bulky hydrophobic Leu 439 and Leu 444. A thorough mutational analysis was undertaken to identify and characterize the transcriptionally critical amino acid residues in VP16C. Assay of the activity of these VP16C mutants on a reporter gene in yeast and in mammalian cells shows that Phe 473, Phe 475, and Phe479 contribute to transcriptional activation, and that hydrophobic side chains are required at these positions. Glu 476 is also involved in activation, but the chemical character of the side-chain at this position is unimportant.

The activation domain of VP16 has been shown to bind to a number of transcription factors *in vitro* including transcriptional adaptor

Ada2 and basal transcription factor TBP. VP16C is both necessary and sufficient for VP16:Ada2 interaction. Assay of the activity of the VP16C mutants on a reporter gene in a yeast strain lacking Ada2, however, failed to identify amino acid residues directly involved in interaction of VP16 with Ada2, suggesting that these proteins do not bind *in vivo*. Fluorescence spectroscopy techniques were applied to study the VP16:TBP interaction. VP16 has been shown to bind TBP and to adopt a more ordered structure in the presence of TBP. Introduction of mutations at positions 442, 475, and 479 that have previously been shown to disrupt transcriptional activation have no effect on binding or TBP-induced structure in VP16. These results suggest that the interaction between VP16 and TBP is not relevant to *in vivo* transcriptional activation by VP16.

To my parents, with love...always

To Glenn and Michelle, my Baby Dinosaur and Baby Frog, who kept me centered by reminding me of the truly important things in life...

To Martin, for tea and sympathy...

"I've been scattered.
I've been shattered.
I've been knocked out of the race, but I'll get better...
And I'll ride the turning world into another night."
- Sting

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However I managed to get here, I certainly did not do it alone!

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When I entered graduate school, I did not have a clear idea of what I was undertaking. Had I known and had I any sense, I might have thought better of it. I will always be grateful to the people who shielded me from my folly and helped me to get through. ☺

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LIST OF ABBREVIATIONS

5-OH-Trp	5-hydroxytryptophan
aa	amino acid
ATP	adenosine triphosphate
bp	base pair
CTD	carboxyl terminal domain of the largest subunit of RNA polymerase II
IPTG	isopropyl-β-D-thiolgalactopyranoside
HEPES	N-2-hydroxyethylpiperiazine-N'-2-ethanesulfonic acid
HPLC	high pressure liquid chromatography
kD	kilodaltons
K_D	dissociation constant
NMR	nuclear magnetic resonance
OD	optical density
PBS	phosphate buffered saline
PolII	RNA polymerase II
RAP	RNA polymerase associated proteins
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAF_{II}	TBP associated factor of RNA polymerase II transcription
TBP	TATA-box binding protein
TFII	transcription factor of RNA polymerase II

VP16	virion protein 16 of herpes simplex virus
VP16C	carboxyl terminal subdomain of the VP16 activation domain
VP16N	amino terminal subdomain of the VP16 activation domain

Amino Acid Codes

Alanine	Ala	A
Argenine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER 1

EUKARYOTIC TRANSCRIPTION BY RNA POLYMERASE II

The process of transcription of messenger RNA from DNA in eukaryotic systems consists of a series of ordered steps: 1) promoter sequences must be recognized and the polymerase complex recruited to or assembled at the promoter, 2) promoter DNA must be unwound to form a complex (the open complex) capable of transcriptional initiation, 3) transcription must be initiated and the first phosphodiester bond synthesized, 4) the polymerase complex must release from the promoter to allow transcription to continue (promoter clearance), 5) the transcript must be synthesized (elongation), and 6) transcription must be terminated when transcript synthesis is complete (197). Any of these steps can be modified by the action of transcriptional activators or repressors to render transcription more or less efficient, respectively.

Transcription of mRNA in eukaryotes is a function of the multi-subunit enzyme RNA polymerase II (PolII). However, PolII alone cannot direct specific transcriptional initiation (274). PolII is joined by general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH to form a complex capable of directing basal transcription (197). Activated transcription requires the action of transcriptional activators, often through the intercession of other proteins known as transcriptional adaptors (259). In this chapter I will provide an overview of the proteins

comprising the basal transcriptional machinery, transcriptional activators and their adaptors, and a model system for the study of transcriptional activation, the VP16 protein of herpes simplex virus.

TRANSCRIPTIONAL MACHINERY

RNA Polymerase II

The RNA polymerase II enzyme of *Saccharomyces cerevisiae* is a multi-subunit complex composed of 12 separate polypeptides, of which only two are non-essential (92). These proteins are highly homologous among species. Three of these polypeptides, Rpb1, Rpb2, and Rpb3 resemble bacterial RNA polymerase subunits β, β' and α, respectively (92). Moreover, at least six of these proteins, including the largest subunit Rpb1, can be functionally replaced in yeast by homologues from higher eukaryotes (1, 181, 236). Electron crystal structures of PolII have been solved (51, 183) (see *Structure and assembly of the preinitiation complex* below).

Perhaps the most curious feature of PolII is the structure of the carboxy-terminal domain (CTD) of its largest subunit. The CTD is conserved among species and consists of tandem repeats of the seven amino acid sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser; the number of repeats appears to increase with the complexity of the organism, ranging from 26-27 in yeast to 52 in human cells (48). The CTD is essential for viability *in vivo*, although deletion of as many as half the heptapeptide

repeats is tolerated in yeast, mouse, and *Drosophila* (12, 190, 276, 291). *In vitro* results suggest that the requirement for the CTD in transcription may depend on promoter structure (1, 116, 127, 157, 230, 257, 291).

Phosphorylation of the CTD results in two forms of RNA polymerase, the unphosphorylated PolIIA and the hyperphosphorylated PolIIO. PolIIA preferentially associates with the preinitiation complex, whereas PolIIO is found in the elongating transcription complex (116, 127, 148, 166). These results suggest that phosphorylation and dephosphorylation of the CTD may be important for the conversion of the polymerase complex from an initiation to an elongation form.

Multiple candidates for the role of *in vivo* CTD kinase have been identified in yeast and cyclin-kinase pairs CTK1/CTK2 (241), Srb10/Srb11 (156), and Kin28/Ccl1 (67, 264). Human homologues of Kin25/Ccl1, the MO15/cyclin H pair, have been identified (231). These cyclin/kinase pairs are subunits of the general transcription factor TFIIH; however, recent *in vitro* evidence indicates that phosphorylation by TFIIH alone is not sufficient to account for the level of CTD phosphorylation observed (261). P-TEFb has also been identified as a CTD kinase in *Drosophila* (174).

Both the Srb proteins and TFIIH directly associate with PolII (97, 137, 198). Moreover, CTD phosphatase activities have been identified in both yeast and human cells, and these proteins are shown to be associated with TFIIF (27-29). The fact that the transcription complex

itself supports both CTD kinase and phosphatase activities provides further support for the importance of CTD-phosphorylation in transcription.

Transcription factor IIA

TFIIA has been isolated and cloned from yeast, human, and *Drosophila* systems (53, 54, 168, 199, 212, 213, 242, 289). Yeast TFIIA is composed of two subunits of 32 kD and 13kD. Human and *Drosophila* TFIIA are composed of three subunits with homology to the yeast proteins. The 37kD α and 19kD β subunits share sequences with the large yeast subunit, while the 13 kD γ subunit is homologous to the small subunit of yeast TFIIA.

TFIIA can bind TBP in the presence or absence of TATA-box DNA, (20, 42, 44, 149, 212) and is capable of increasing the affinity of TBP for DNA (107). Stimulation of activation by VP16 has been shown to require all three subunits of human TFIIA (167). X-ray crystal structures of two TBP-DNA-TFIIA complexes have been solved (77, 249)(see *Structure and assembly of the preinitiation complex* below).

Transcription factor IIB

TFIIB is a single polypeptide that has been identified in yeast (38kD), *Drosophila* (34 kD) and human (35kD) systems (88, 206, 270, 285). TFIIB has been shown to bind both the TFIID/DNA complex (20,

171), and TFIIF/PolII (64, 89), suggesting that it may function as a link between TFIID bound to DNA and the rest of the transcriptional machinery. TFIIB has also been implicated in start site selection (155, 206) and has recently been demonstrated to be a sequence specific DNA binding protein (146).

TFIIB consists of two domains, an N-terminal cysteine rich region and a C-terminal protease resistant region containing two 75 amino acid imperfect repeats (10, 172). The NMR structure of this TFIIB core region (TFIIBc) (9) and an X-ray crystal structure of TFIIBc-TBP-DNA complex have been solved (189) (see *Structure and assembly of the preinitiation complex* below).

Transcription Factor IID

TFIID was originally identified as a *Drosophila* nuclear extract component capable of binding to the TATA-box DNA sequence of class II promoters (201). Subsequent work from many laboratories has determined that the TATA-box binding activity resides in a single polypeptide, the TATA-box binding protein (TBP). TBP exists in association with multiple other factors (TAFs) to comprise the TFIID complex.

TATA-box binding protein

The cDNA for TBP was initially cloned from yeast (91, 104). Subsequently homologous proteins have been found and the genes

cloned from *Drosophila*, mouse, and human cells (101, 117, 186, 205, 248). In plant species, two distinct TBP genes have been identified (74, 90). TBP proteins range in size from 22 kD in *Arabadopsis* to 38 kD in human. The carboxy-terminal 180 amino acid region of TBP contains two direct repeats, and this core region is conserved among species. The N-terminal region is more variable. Crosslinking and affinity chromatography experiments suggest that TBP exists as a dimer in solution (41).

The primary function of TBP is promoter recognition through binding to the TATA-box. X-ray crystal structures for TBP bound to DNA have been solved (122, 128). X-ray crystallography, photo-crosslinking, and mutational analysis have also provided structural information about TBP complexed with DNA and other basal transcription factors (77, 147, 189, 216, 251) (see *Structure and assembly of the preinitiation complex* below). TBP is also integral to the activity of RNA polymerase I and RNA polymerase III (100).

TBP-associated factors

Genes encoding the TAFs have been cloned from yeast (yTAF$_{II}$ 150,130/145, 90, 68/61, 67, 60, 47, 40, 30, 25/23, 19, 17), *Drosophila* (dTAF$_{II}$ 250/230, 150, 110, 80/85, 60/62, 40/42, 30α/28/22, 30β), and human (hTAF$_{II}$ 250, 130, 100, 80/70, 68, 55, 30, 29, 31/32, 20/15, 18, and the B-cell specific hTAF$_{II}$105) systems (253). Six of these proteins are homologous across all three species, while another four are shared

between two of the three species. Multiple TAF-TAF and TBP-TAF interactions have been demonstrated (21), as has the ability of multiple TAFs to bind to activation domains (see *TAFs* below). Although essential for viability, TAFs are not essential for all transcription in eukaryotes (253, Moqtaderi, 1996 #398, 269). Recently, certain yTAF$_{II}$s (90, 68/61, 60, 25/23, 17) have been shown to be a part of the yeast SAGA nucleosome acetylation complex (86), and human, *Drosophila*, and yeast TAF$_{II}$250 have been shown to have histone acetyltransferase activity *in vitro* (185), providing a link between TAFs and chromatin modification.

Transcription factor IIE

In human cells, TFIIE exists as a heterotetramer composed of two copies each of a 56 kD and a 34 kD subunit (110, 195, 204). Yeast (66 kD and 43 kD) and *Drosophila* (55 kD and 38 kD) homologues have also been identified (66, 273). TFIIE has been shown to bind directly to the non-phosphorylated form of RNA polymerase II, TBP and TFIID, both subunits of TFIIF and TFIIH. TFIIE is functionally associated with TFIIH (194) and has been shown to promote phosphorylation of the CTD by TFIIH (229), and to be involved with TFIIH in open complex formation and promoter clearance (84, 102, 141).

Transcription Factor IIF

TFIIF has been cloned from yeast (99), *Drosophila* (71, 80, 81, 119), and human systems (7, 68, 239). Mammalian TFIIF is composed of RAP30 (30kD) and RAP74 (58 kD); the *Drosophila* protein is homologous. Yeast TFIIF consists of three subunits, the larger of which (105 kD and 54 kD) are also homologous to RAP74 and RAP30, and the smallest of which is $yTAF_{II}30$.

TFIIF recruits PolII to the preinitiation complex through RAP30 (69). RAP30 also shares regions of homology with bacterial sigma factors (178, 239) and these findings together suggest a role for RAP30 as an initiation factor important in promoter recognition (30, 120, 121). RAP74 also appears to function in initiation (152, 250).

TFIIF has also been implicated in elongation (13, 208). RAP74 is essential for this function (30, 152) and RAP30 may also contribute (250). TFIIF activity in initiation and elongation can be regulated by phosphorylation of RAP74 (131). $TAF_{II}250$ has been shown to be a serine kinase and to selectively phosphorylate RAP74 and not other basal transcription factors (55). Like the PolII though the action of CTD kinases, TFIIF may be altered from initiation to elongation factor by $TAF_{II}250$ phosphorylation.

Transcription factor IIH

TFIIH is the only transcription factor to exhibit multiple enzymatic activities. TFIIH is capable of ATPase and helicase activities, as well as phosphorylation of the CTD of PolII (58). Mammalian TFIIH is a multimeric protein of eight polypeptides ranging from 34-89 kD (58). Two of these subunits have been identified as MO15 and cyclin H, the kinase/cyclin pair responsible for CTD phosphorylation by TFIIH (231). *Drosophila* TFIIH has also been purified and is similarly composed of eight polypeptides in the range of 30-100 kD (8). TFIIH in yeast comprises nine polypeptides (244), two of which are the CTD kinase/cyclin pair Kin28/Ccl1 (67, 264).

As described above, TFIIH has been shown to phosphorylate the CTD of PolII, and to act in open complex formation and promoter clearance, all in association with TFIIE. TFIIH has also been shown to function in transcriptional elongation (61, 288) and in nucleotide excision repair (NER) of DNA lesions (57, 225). However, only a subset of TFIIH subunits are also found in the NER complex and the two activities of TFIIH appear to be functionally independent (222, 246).

Structure and assembly of the preinitiation complex

In the past several years, a wealth of data has become available regarding the structure of the preinitiation complex. X-ray crystallography has established the configuration of TBP-TATA, TBP-

TATA-TFIIA, and TBP-TATA-TFIIB complexes (77, 122, 128, 189, 249). Electron crystallography has produced a structure for RNA polymerase II and a PolII-TFIIB-TFIIE complex (51, 154, 183). Photocrosslinking and mutagenesis have determined the relative locations of DNA, PolII, TBP, TFIIA, TFIIB, a subunit of TFIIE, and TFIIF (45, 70, 124, 147, 216, 251). Based on these results, the following model of preinitiation complex structure can be described.

The preinitiation complex centers on the interaction of the saddle-shaped TBP with TATA-box DNA. TBP induces an approximately 80° bend in the DNA at the TATA-box which results in binding of the minor groove of the TATA-box to the concave surface of the TBP. TFIIA binds to the N-terminal stirrup of TBP and contacts the DNA backbone immediately upstream of the TATA-box. TFIIA may have additional contact with DNA downstream of TATA. TFIIB binds to the underside of the TBP-TATA complex and its repeats make asymmetric contact with DNA both upstream and downstream of the TATA-box. TFIIB binds to the C-terminal stirrup of TBP. Both TFIIE and TFIIF contact DNA downstream of the TATA-box but upstream of the transcriptional start site. RNA polymerase II contacts DNA both upstream of the TATA-box and downstream beyond the transcriptional start site, and appears to induce a second bend (≈70°) in the DNA at the start site that results in wrapping of the DNA around the polymerase. The locations of the other components of TFIID and TFIIH have not been determined.

Although experimental data generally agree on a structure for the preinitiation complex, the method of assembly of the transcriptional apparatus on DNA is controversial. The traditional model for preinitiation complex assembly, derived primarily from *in vitro* experiments with purified factors, is stepwise construction (210). First, TFIID binds the TATA-box through TBP, an interaction stabilized by TFIIA. Then TFIIB joins to form the DAB complex. TFIIF escorts PolII into this complex, forming the DABPolF complex. This is followed by addition of TFIIE and TFIIH to form the complete preinitiation complex. More recently, however, it has been suggested that *in vivo* PolII and its associated factors arrive at the promoter in a preformed complex, the holoenzyme (256).

RNA polymerase II holoenzyme complexes were originally isolated in yeast and found to contain PolII, TFIIF, and Srb proteins with or without TFIIB and TFIIH (129, 137, 255). As with the preinitiation complex assembled sequentially, these complexes were found to require TFIIB, TFIIE, TFIIH, and TBP to initiate transcription *in vitro*. An additional pair of Srb proteins that comprise a kinase/cyclin pair, and proteins of the SWI/SNF chromatin remodeling complex have also been shown to be part of the holoenzyme (156, 278). More recently a distinct holoenzyme complex has been isolated which contains PolII, TFIIB, TFIIF, Gal11, Cdc73 and Paf1, but does not contain the Srb proteins (234). Mammalian forms of holoenzyme have also been isolated (37, 198) and

the human form has been shown to contain a SWI/SNF subunit (188). The assembly and holoenzyme models are not necessarily mutually exclusive; it seems likely that a holoenzyme would be assembled in a stepwise fashion. Either model results in an assembled preinitiation complex that is competent for transcription.

TRANSCRIPTIONAL ACTIVATION

Transcriptional activators cause an increase in the level of transcription by RNA polymerase II; however, their mechanism of action is unclear. Activators have been proposed to recruit chromatin remodeling enzymes to allow promoter recognition and pre-initiation complex formation (130). Likewise, activators may recruit basal transcription factors to facilitate pre-initiation complex formation (209), increase initiation rate or aid in promoter clearance (259), or increase the rate of elongation (14). The structure and mechanism of action of transcriptional activators has been the subject of intense study and debate.

Transcriptional activators

Proteins that activate transcription are typically composed of two functionally distinct domains, one that facilitates gene specific association with DNA at the promoter and the other that performs the activation function (290). Activation domains can often be further

divided into subdomains capable of independent transcriptional activation. This has been demonstrated for activator proteins such as Gal4 (169), Gcn4 (59, 103), Rta (93), Zta (35, 184), Fos and Jun (243), RelA (16), and VP16 (83, 214, 228, 260, 268).

Activators have traditionally been classified according to the most prevalent amino acid of their activation domain. Thus activators have been termed acidic, glutamine-rich, proline-rich, or serine/threonine-rich (259). However, mutational analysis of a number of activation domains including VP16 (47, 214), RelA (16), p53 (160), Bell (151), GCN4 (111), the glucocorticoid receptor (2, 109), C1 (221), NRF-1 and NRF-2(87), and Sp1 (79), has demonstrated that bulky hydrophobic and aromatic amino acids are more critical for activation function than those that are most abundant.

Because activation domains in isolation appear highly unstructured (56, 193, 226, 232, 265), structural information has been difficult to obtain. Recently, however, several laboratories have reported structures of activation domains induced by the presence of putative target proteins. The activation domain of the herpes simplex virus protein VP16 has been demonstrated to adopt a more constrained structure in the presence of TBP (233). In the presence of $hTAF_{II}32$, a portion of the VP16 activation domain adopts an α-helical structure (263). Secondary α-helical structure is also induced in an activation domain of CREB by a portion of CBP (211), the transcription factor c-myc

in the presence of TBP (179), and the p53 transcriptional activation domain in the presence of its repressor MDM2 (143). The structural data support the results of the mutational analyses, as the interaction surfaces were composed principally of hydrophobic residues.

Virion Protein 16

The VP16 activation domain is a common model system in the study of transcriptional activation. VP16 (also known as Vmw65 or α-TIF) is an immediate early gene activator of herpes simplex virus. VP16 is a structural component of the virion particle that is released into the cell and migrates to the nucleus upon infection (191). VP16 as a structural component is essential for viral replication (275); however, the transcriptional activation function of VP16 is not required for viability (237); R.Pichyangkura and S.J.Triezenberg, unpublished). VP16 is not capable of binding DNA (173, 180), but associates with the promoter in complex with two cellular proteins, the sequence specific DNA-binding protein Oct-1 (3, 78, 140, 192, 207, 240) and HCF (78, 118, 139, 284). The phosphorylation state of VP16 is critical for the formation of this complex (196).

VP16 is a 490 amino acid protein that can be divided into two domains (95, 260). The amino terminal 410 amino acids comprise the DNA association domain and are responsible for complex formation with

Oct-1 and HCF (191). The carboxyl terminal 80 amino acids of VP16 are necessary and sufficient for transcriptional activation both in the virus and when fused to a heterologous DNA-binding domain (46, 220, 260). The VP16 activation domain can be further divided into two subdomains, VP16N (amino acids 412 to 450) and VP16C (amino acids 450 to 490), each of which is independently capable of activating transcription when provided with a DNA binding mechanism (83, 214, 228, 260, 268). Extensive mutational analysis of VP16N has indicated that the aromatic character of F442 and the bulky hydrophobic nature of residues L439 and L444 are critical for transcriptional activation by this subdomain (47, 214). Mutational analysis of VP16C described in this thesis indicates that the bulky hydrophobic nature of residues F473, F475, and F479 are critical for activation by the C-subdomain (Chapter 3). Glutamate 476 also contributes to activation, but not through a side-chain interaction.

Activator mechanisms

1. Chromatin remodeling

Chromatin mediated repression of transcription can be alleviated by the addition of transcriptional activators (114, 164, 279). Histone acetylation has also been shown to be involved (266). Complexes capable of disrupting histone structure and allowing promoter recognition may

function as transcriptional activators themselves, or can be recruited by other proteins.

In yeast, several such complexes have been identified including the SWI/SNF complex (22, 203) and its homologous complex RSC (23). SWI/SNF complexes have also been identified in mammalian systems (145). SWI/SNF disrupts but does not dislodge histones and has been shown to facilitate both activator binding and TBP binding to promoter DNA (43, 106, 138).

More recently, histone acetylation as a means of chromatin remodeling has come to the fore. In yeast, the SAGA complex (85) and the ADA complex (105) are transcriptional adaptors share a histone acetyltransferase, Gcn5, whose activity is required for function of both complexes (26, 85, 142, 271). Homologues of Gcn5 have also been identified in human (24, 286), *Drosophila* (238), and *Tetrahymena* (19). Human homologue PCAF is also a histone acetyltransferase, and is associated with three other such proteins, p300, CBP, and ACTR1 (32). The acetyltransferase activity of CBP has also been demonstrated to stimulate transcription (175). Homologues of Ada2, another member of the ADA complex, have also been found in the human system (24).

Ada2 has been shown to be required for maximal transcriptional activation by Gcn4 (59, 60), Bell (15), p53 (25), retinoid X and estrogen receptors (267), and the tau1-core domain of the glucocorticoid receptor (98), and the activation domain of VP16 (235). Yeast activator Adr1 has

also been shown to bind Gcn5 (36). CBP and p300 have been shown to interact with activators such as CREB (4, 144), E1A (286), and nuclear receptors (115), and PCAF is also a nuclear receptor coactivator (17). These activation domain interactions may be important for recruiting the histone acetyltransferase complex to the promoter.

These observations are also consistent with a holoenzyme model for preinitiation complex formation. The yeast RNA PolII holoenzyme has been found to remodel chromatin (76). Proteins of the SWI/SNF chromatin remodeling complex have been shown to be part of the holoenzyme in yeast (156, 278) and human forms (188). CBP and p300 have also been found to be associated with human TBP (49).

2. Recruitment of general transcription factors

a. TFIIA

The presence of TFIIA has been shown to increase the levels of transcription activated by such proteins as VP16, Zta, Sp1, NTF-1, the synthetic activator Gal4-AH, and SV40 large T antigen (50, 199, 242, 289). Moreover, Zta, VP16, and T antigen bind directly to TFIIA, and this binding correlates with the ability to stimulate TFIIA-TFIID-TATA complex formation (50, 134, 135, 199). These results suggest that the certain activators recruit TFIIA to the promoter to promote DA complex assembly, although this may not be the case for all promoters (158).

Isomerization of a TFIIA-TFIID-TATA complex into a form that is capable of binding TFIIB has been shown to be necessary for activation of transcription (34). It has been suggested that the role of TFIIA is to assist in overcoming the slow step of TBP binding in preinitiation complex formation (35). Taken together, these results suggest that the role of the transcriptional activator might be to recruit TFIIA and through that recruitment to speed preinitiation complex formation.

b. TFIIB

Several transcriptional activators have been shown to interact with TFIIB. The synthetic activator Gal4-AH, CTF1, and the activation domain of VP16 have been shown to bind TFIIB and recruit it to the promoter (39, 126, 161, 218). Correlation between binding to TFIIB and *in vivo* activity has also been demonstrated for yeast Gal4 protein (280) and RXRβ in mouse (153). However, the model of activator recruitment of TFIIB to the promoter is controversial. Recruitment of TFIIB to the promoter was initially proposed as a function of VP16 to overcome the rate limiting step in preinitiation complex formation (161). However, a study of VP16 indicated a requirement for more than interaction with TFIIB to obtain full activation (268). Furthermore, mutations in TFIIB that disrupt the stability of the TBP-TATA-TFIIB complex do not affect the response of these complexes to activation by VP16 (38). Experiments with TFIIB tethered at the promoter via fusion to a DNA binding domain

have suggested that recruitment of TFIIB is not the rate limiting step in complex formation (82).

Comparison of the solution structure of IIBc (9) and the TBP-TATA-TFIIB x-ray crystal structure (188) indicates a change in the conformation of the TFIIB repeats in the presence of TBP and DNA. Roberts *et al.* had previously demonstrated that VP16 is capable of inducing a conformational change in TFIIB by binding to the carboxyl terminal domain (217). This result has been confirmed recently by NMR (94). These results suggest a role for VP16 and other activation domains not in TFIIB recruitment, but in alteration of TFIIB conformation to facilitate binding of TFIIB to the TBP-DNA complex.

c. *TFIID*

i. *TBP*

In addition to binding DNA, TBP has been shown to bind *in vitro* to transcriptional activators such as VP16 (108), Sp1 (63), Oct-1 and Oct-2 (293), Gal4 (182), c-Jun and other members of the c-Jun family (72), E2F1 (202), p53 (163, 262), ERM (52), and E1A (162). Moreover, mutational analysis of TBP has allowed identification of TBP residues that disrupt activator function *in vivo* (5, 123, 125, 150, 159, 254). Several functions for these interactions have been proposed. Recruitment of TBP to the TATA-box may be assisted by transcriptional activators. By tethering TBP to the promoter through a DNA binding domain, the requirement for an activation domain can be relieved (31,

132, 170, 281). However, mutational analysis and tethering experiments indicate that while this may be the function of some activation domains, such as Sp1, it may not be relevant to the function of others (96, 252).

Experimental evidence suggests that both TBP and TFIID can exist as dimers in solution, raising the possibility that the function of certain transcriptional activators with respect to TBP is to dissociate the TBP dimer, allowing TBP to interact with TATA-box DNA (40, 41, 247). A mutation in TBP that disrupts transcriptional activation by VP16 was found on the DNA binding surface of TBP (123). VP16 has also been suggested to induce a conformational change in TBP that affects binding of TBP to TATA (159).

ii. TAFs

As with TBP binding, binding of activators to TAFs may recruit TBP/TFIID to the promoter. The TAFs were originally identified as proteins that mediate transcriptional activation (62). Many transcriptional activators are capable of binding to TAFs. VP16 binds dTAF$_{II}$40 and its human homologue hTAF$_{II}$32 (83, 133). Sp1 binds both dTAF$_{II}$150 and dTAF$_{II}$110 (33, 79), and NTF-1 binds dTAF$_{II}$150 (33). Progesterone receptor and Bicoid also bind dTAF$_{II}$110 (223, 227), and Bicoid binds d TAF$_{II}$60 as well (223). ERM, a member of the ETS family, binds both dTAF$_{II}$60 and dTAF$_{II}$40 (52). Adenovirus activator E1A interacts with hTAF$_{II}$135 (177). Moreover, these activities are specific; for example, the estrogen receptor binds hTAF$_{II}$30 while VP16 does not (112).

There is evidence that at least some of the interactions between activators and TAFs occur *in vivo* (224).

d. TFIIE and TFIIF

Although both TFIIE and TFIIF have been shown to be involved in both initiation and promoter clearance (see above), they do not appear to be particularly targeted by transcriptional activators. Epstein-Barr Virus transactivator EBNA 2 has been shown to interact with TFIIE through a coactivator, p100 (258). TFIIF has been shown to play a role in transactivation by serum response factor and VP16, both of which facilitate the binding of the RAP74 subunit to DNA (292).

e. TFIIH

TFIIH has been shown to bind to the activation domains of both VP16 and p53 (283). However, the correlation between the ability of VP16 to bind to TFIIH and to activate transcription seems to be restricted to the VP16N subdomain (135, 283). Recently, a connection between several activation domains and the kinase activity of TFIIH has been demonstrated. The RARα activation domain AF-1 can bind to TFIIH and be phosphorylated by the TFIIH kinase both *in vivo* and *in vitro* (219). This phosphorylation is required for *in vivo* activation by RARα. Likewise, the activation domain of p53 can be phosphorylated by the TFIIH kinase *in vitro* and this enhances the DNA binding activity of p53

(165). The HIV-1 transactivator Tat has also been found to bind TFIIH kinase *in vitro* and *in vivo*, but in this case Tat stimulates phosphorylation of the PolII CTD by TFIIH (73). The link between TFIIH and transcriptional activation domains may involve either protein modifiying the action of the other.

f. Recruitment and the holoenzyme

The majority of the data discussed above, although previously considered solely in light of the stepwise assembly model, is compatible with the holoenzyme model as well. Known targets of activators such as TFIIB, TFIID, TFIIE, TFIIF, and TFIIH have been found in various holoenzyme complexes (37, 129, 137, 198, 255). The activation domains of VP16 and Gal4 have been shown to bind to the PolII holoenzyme in yeast (97, 136). Moreover, recruitment of the PolII holoenzyme complex to a promoter by tethering it via fusion of a holoenzyme protein to a DNA binding domain has been shown to be sufficient for activation of transcription (65, 75).

3. Open complex formation

The transition of the polymerase complex from a preinitiation form to an elongation competent form is known as open complex formation. The duplex promoter DNA is separated to allow access to the template strand in an ATP-dependent manner (272). VP16 and HIV Tat activator

have been shown to influence transcription beyond the step of preinitiation complex formation (277, 282). Mutations in VP16 have also been shown to affect the formation of the open complex (113). Both TFIIE and TFIIH have been shown to be required for open complex formation (102). Thus activators may recruit TFIIE, TFIIH, or some other component to stimulate open complex formation.

4. Promoter clearance

The transition from an open complex to an actively elongating complex is known as promoter clearance and involves formation of the first phosphodiester bond in the nascent mRNA chain. This step seems to require both TFIIE and TFIIH, and may involve phosophorylation of the PolII CTD (61, 84, 176). Only one activation domain, that of the human c-AMP response element binding protein PBP, has been demonstrated to increase the rate of promoter clearance (187). However, activation domains that interact with TFIIE and TFIIH could stimulate promoter clearance through recruitment of these factors.

5. Elongation

The polymerase elongation complex differs from the initiation complex. The elongating polymerase is found in the CTD-phosphorylated form, PolIIO, and the complex includes elongation factors such as TFIIS and SIII (also called elongin) (6, 215), but not adaptor complexes such as

mediator (245). TFIIF and TFIIH are also important for elongation (13, 208, 288). The transcriptional activators VP16, E1a, Tat, p53, and E2F1 have all been shown to stimulate elongation (18, 287), possibly through interaction with these transcription factors or with elongation factors. These observations are consistent with the holoenzyme model, as both TFIIS and a subunit of SIII have been found to be associated with a human PolII holoenzyme (200).

SUMMARY AND OUTLINE

Transcriptional activation is a complex process that is not yet well understood. General transcription factors have been cloned and characterized. Structural information has provided a partial picture of the assembled transcriptional machinery. The function of adaptor complexes in chromatin remodeling has been more clearly defined. However, the mechanisms by which transcriptional activators regulate this system, especially *in vivo*, remain ill-defined. The purpose of this thesis research is to provide a better understanding of transcriptional activation through the study of a model transcriptional activator, VP16.

The activation domain of VP16 can be divided into two subdomains, the N-terminal of which has previously been characterized (47, 214). I have undertaken a thorough mutational analysis of the VP16C subdomain fused to a heterologous DNA binding domain to identify and characterize amino acid side-chains critical for activation by

VP16C. The VP16C mutants were assayed for the ability to activate reporter genes in both yeast and mammalian cells. The results of these experiments, described in Chapter 3, indicate that the side chains of three bulky hydrophobic residues – F473, F475, F479 – play critical roles in VP16C activation in both systems, and that E476 also contributes to activation, though not through a side-chain specific interaction.

The results of the mutational analysis were then employed to explore the interactions of VP16 with two of its putative target proteins. The activation domain of VP16 has been shown to bind *in vitro* to a number of transcription factors (11, 83, 108, 133, 134, 161, 283). One of these targets, the yeast protein Ada2, has been shown *in vivo* to be required for maximal activation by the VP16 activation domain (235) and to bind VP16 *in vitro* through VP16C (11, 235). To identify amino acid residues in VP16C required for interaction with Ada2, the VP16C mutants were assayed for reporter gene activation in a yeast strain lacking Ada2. These experiments, described in Chapter 4, failed to identify any amino acid side-chains directly involved in interaction with Ada2.

The activation domain of VP16 has also been shown to bind TBP (108). Fluorescence spectroscopy has been applied to study this interaction and has shown that the VP16 activation domain becomes structurally constrained, and that amino acid residues F442 and F473 are shifted from a solvent exposed to an hydrophobic environment in the

presence of TBP (232, 233). To explore the connection between the amino acid residues that are critical for transcription *in vivo* and the interaction between VP16 and TBP, further fluorescence studies were undertaken in which VP16 activation domains bearing transcriptional mutations were analyzed in the presence of TBP. The theory of fluorescence spectroscopy is described in Chapter 2. The results of these experiments, described in Chapter 5, indicate that these mutations produce no change in the TBP-induced structure of VP16. Preliminary experiments with a wild type VP16 activation domain and TBP in the presence of TATA-box DNA, however, indicate that TATA-box DNA causes TBP to dissociate from VP16.

The results of my thesis research provide some insight into the transcriptional activation domain of VP16. Moreover, they provide a thorough panel of mutants and a well-defined biophysical technique to employ in further study of VP16. In Chapter 6, I outline future experiments of this nature, as well as suggestions for application of the mutational data to VP16 in its native context, herpes simplex virus.

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CHAPTER 2

INTRODUCTION TO FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy makes use of the physics of light energy transfer to provide information about a molecule. It is an especially useful technique for describing the environment of the fluorescing moiety, or fluorophore. The degree of solvent exposure of the fluorophore, the hydrophobicity of its surrounding environment, the degree of order in its surrounding environment, and changes induced in any of these parameters by the presence of other compounds can be determined. Through the use of a fluorescent amino acid, fluorescence spectroscopy can be used to study the environment of a particular residue in a protein. Thus, fluorescence spectroscopy is an ideal tool for the study of protein interactions.

Basic Principles of Fluorescence (20, 21)

Absorption of a photon of light by a fluorescent molecule results in the excitation of electrons. Observed fluorescence is the emission of energy in the form of light as electrons relax from the first excited state, S_1, to the ground state, S_0. The basic principles of fluorescence are diagrammed in Figure 1.

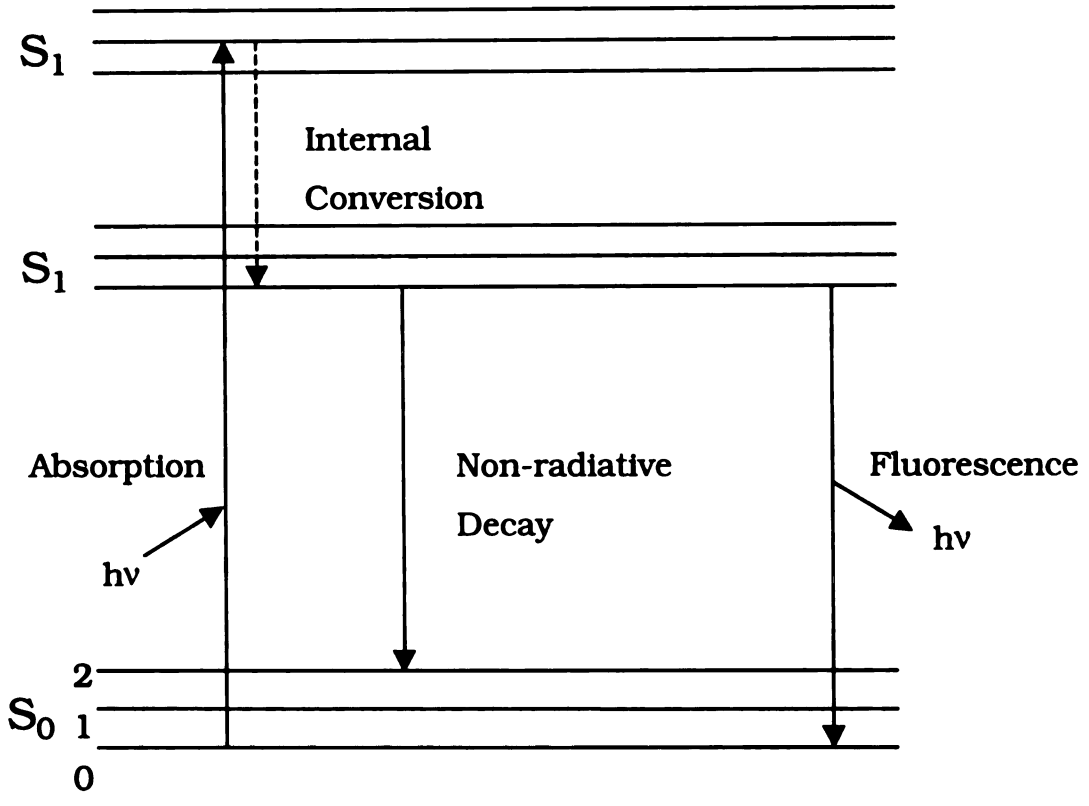

Figure 1. Jablonski diagram of the absorption and emission of light energy. The ground state is denoted by S_0 and the first excited state is denoted by S_1. hν represents a photon of light.

As described by the Franck-Condon principle, light absorption is practically instantaneous; absorption of a photon of light takes place in approximately 10^{-15} seconds. Absorption is followed immediately by a relaxation of the excited electrons to the lowest vibrational state of the S_1 energy state. This process, known as internal conversion, is also extremely rapid, requiring on the order of 10^{-12} seconds. From the lowest vibrational level of the S_1 state, some energy is dissipated through a variety of non-radiative decay processes such as molecular collisions, rotational or translational diffusion, or formation of molecular complexes. The energy from the S_1 electrons can also be released as emitted light: fluorescence.

Fluorescence emission is variable. Any given fluorophore has both a lifetime and a quantum yield. The fluorescence lifetime of a fluorophore is the average time that its electrons remain in the excited state prior to a return to the ground state. It is defined as:

$$\tau = \frac{1}{\Gamma + k} \tag{1}$$

where Γ is the rate of fluorescence emission and k is the sum of the rates of all non-radiative decay processes (Figure 1). Fluorescence lifetimes are on the order of 10^{-8} seconds (10ns). Quantum yield of a fluorophore is defined as:

$$Q = \frac{\Gamma}{\Gamma + k} \tag{2}$$

Fluorescence emission properties such as lifetime and quantum yield depend on the chemical structure of the fluorophore. Most fluorescent molecules contain a conjugated double bond system; as such, aromatic organic molecules are common fluorophores.

Instrumentation For Fluorescence Spectroscopy (20, 21)

Measurement of fluorescence requires substantially the same instrumentation regardless of whether steady state or time resolved measurements are being made. A basic plan of a fluorescence spectrophotometer is shown in Figure 2. The excitation source provides a beam of light that is passed immediately through a monochrometer to allow selection of the excitation wavelength. A portion of this excitation beam may be shunted to a reference cell to allow correction for variations in excitation intensity. The remainder of the excitation beam passes through the sample, exciting the fluorophore therein. The emitted light is monitored at a right angle to the excitation because this angle minimizes the effects of scattered light on the measured fluorescence. The emission beam is passed through a second monochrometer where emission wavelength is selected, and then reaches the detector. From the detector the information is sent to a recorder. Polarizers in the path of excitation and emission beams are generally removable. These are necessary for experiments such as fluorescence anisotropy that require

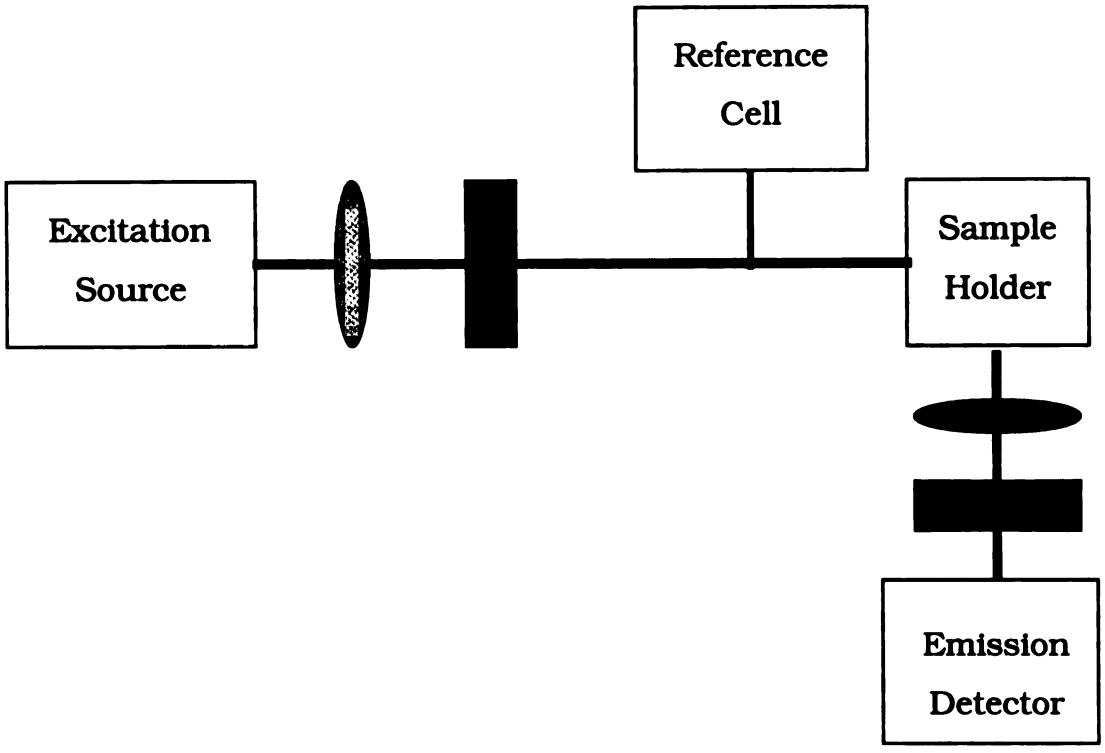

Figure 2. Schematic representation of an L-format fluorescence spectrophotometer. Grey ovals represent monochrometers. Black rectangles represent optional polarizers.

plane polarized light (see below). Excitation and emission monochrometers, and polarizers are typically under computer control.

Either a single or a double detector arrangement may be used in a fluorescence instrument. The single L-format is the simplest and most common detection for most steady state applications. For fluorescence anisotropy experiments, the sample is excited with vertically polarized light and both the vertically polarized emission and the horizontally polarized emission are recorded. In these types of experiments, a two detector system is preferred, as it eliminates the need to continually shift the emission polarizer between vertical and horizontal settings. Each detector is placed at 90° to the sample and the resulting arrangement is referred to as the T-format.

Steady state fluorescence spectroscopy employs a constant excitation beam. While the presence of a reference cell does correct for variation in beam intensity, the preferred excitation source for steady state instruments is one which produces a steady beam. High pressure xenon arc lamps are the steadiest of the sources available and therefore the most commonly used. They also provided excitation over a range of wavelengths, which makes them useful for exciting a variety of fluorophores. The detector in a steady state instrument is almost invariably a photomultiplier tube (PMT).

Time resolved fluorescence spectroscopy requires a pulsed excitation beam. Sources are selected for their ability to create short

pulses, consistent pulse intensity, and to be tunable to multiple wavelengths. The system which best combines all of these factors is a synchronously pumped mode-locked dye laser. This system uses a high energy laser which is capable of very brief, stable pulses to excite a dye laser. Dye lasers are tunable within the wavelength range of the dye employed. Moreover, the dye can be changed to alter the available wavelengths, rendering this system extremely versatile in terms of analyzing multiple fluorophores. The most highly used and sensitive detection system is time correlated single photon counting (TCSPC). TCSPC detects the time between the excitation pulse and the arrival of the first photon at the detector as a digital signal. Time resolved decay data are obtained in the form of a histogram of number of photons versus arrival time. Data collection continues until a certain number of photons are obtained; reliable data generally require on the order of 15,000 counts (18).

Tryptophan: An intrinsic protein fluorophore (20, 21)

Two classes of fluorophores are used in fluorescence spectroscopy studies. Fluorophores which are external to the molecule under study are known as extrinsic probes. They can be added covalently or non-covalently to the subject molecule. Fluorophores which are part of the molecule under study are called intrinsic probes.

In the study of protein, three intrinsic probes are theoretically available. The amino acids phenylalanine, tryptophan, and tyrosine all fluoresce. However, the quantum yield of phenylalanine is very low and although the quantum yield of free tyrosine is high, tyrosine fluorescence in proteins is weak. Tryptophan typically accounts for 90% or greater of the total fluorescence of a given protein. For the purposes of fluorescence spectroscopy, tryptophan can be excited at wavelengths which do not excite either phenylalanine or tyrosine, so that tryptophan accounts for 100% of the observed fluorescence. Moreover, the quantum yield of tryptophan is high enough that the signal from a single tryptophan residue in a protein is sufficient for most experimental purposes.

The presence of multiple tryptophan residues in a protein complicates interpretation of fluorescence data. Unless the environments of the various tryptophan residues are significantly different, the signals cannot be separated. Data of this type can only represent the average of the signals from the various tryptophans. Study of a unique tryptophan, in contrast, allows study of a protein from a clear, single perspective. Because tryptophan is a relatively rare amino acid in proteins, it is frequently a simple matter through the use of site-directed mutagenesis techniques to engineer a protein with a unique tryptophan probe for study. Not only does this simplify the signal, it allows the deliberate placement of probes at a variety of positions in the

protein so that the protein can be studied from multiple perspectives. In order that the results of these experiments be representative of the wild type protein, however, care must be taken to ensure that tryptophan mutations do not disrupt the normal activity of the protein.

Tryptophan is rare in protein composition, but it is not entirely uncommon. Large proteins will almost certainly have more than one tryptophan residue. For the purposes of protein interaction studies in which more than one protein is present, then, use of mutagenesis to remove all but a single tryptophan probe in a single protein is impractical. However, there are multiple analogues of tryptophan with fluorescence properties that differ from those of native tryptophan. The absorbance of 5-hydroxytryptophan and 7-azatryptophan, for example, is red-shifted compared to that of native tryptophan. These analogs can be selectively excited in the presence of normal tryptophan such that the fluorescence signal will derive solely from the analog not from the tryptophan. Conversely, the absorbance of the 4-fluorotryptophan analog is blue-shifted relative to that of tryptophan. The absorbance of this analog at the normal excitation wavelengths for tryptophan is less than 1% of the absorbance of native tryptophan. Proteins that incorporate the 4-fluorotryptophan analog can therefore be rendered invisible in the presence of normal tryptophan. Use of site-directed mutagenesis techniques in conjunction with selective incorporation of

tryptophan analogs allows spectroscopic studies to focus on a given position of a single protein in the presence of multiple species.

Steady State Emission Spectra (20, 21)

Steady state emission spectra describe the emission intensity of a fluorophore at multiple wavelengths. Emission spectra can be altered by changes in the local environment of the fluorophore, such as accessibility to solvent and hydrophobicity. Tryptophan in aqueous solution has an emission maximum of 348nm (35). Tryptophan in a non-polar environment such as the hydrophobic core of a protein would show a blue shift, the emission maximum would move to a shorter wavelength. The degree of shift indicates the accessibility of the tryptophan to contact with the solvent molecules, providing information as to whether the tryptophan is buried within the protein or exposed on its surface.

Fluorescence Anisotropy (20, 21)

Fluorophores preferentially absorb photons with an electric moment parallel to the transition moment of the fluorophore. If a random solution of a fluorophore is excited with plane polarized light, this results in the selective excitation of those fluorophores with a transition moment in that plane. Likewise, the emission of these excited fluorophores is partially polarized. These polarized emissions can be recorded and an anisotropy value can be calculated:

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}} \quad (3)$$

where I_{II} is the vertically polarized emission and $I_{\perp}$ is the horizontally polarized emission when the excitation is vertically polarized.

Anisotropy results from the angle between the absorption transition moment and the emission transition moment for a given fluorophore. However, the absorption transition moment need not be exactly parallel to the excitation photon for excitation to occur. The probability of excitation of a given fluorophore in a randomly oriented population is proportional to $\cos^2\theta$ where θ is that angle between the absorption dipole and the excitation axis. The mathematical consequence of this probability is that the theoretical maximum anisotropy for any given species is 0.4.

Experimentally, many factors can combine to decrease measured anisotropy values from the 0.4 limit. Some of these are sources of experimental error such as light scattering, reabsorption of scattered light, improperly aligned polarizers, or energy transfer between molecules in a dense solution. In an optimized solution with a sufficiently dilute fluorophore, however, the major cause of decreased anisotropy is rotational diffusion of the fluorophore. It is for this reason that anisotropy measurements are valuable.

For a system in which rotational diffusion is the only significant contributor to changes in anisotropy, anisotropy can be defined as:

$$r = \frac{r_0}{1 + \left(\frac{\tau}{\phi}\right)} \quad (4)$$

where r_0 is the anisotropy value in the absence of rotational diffusion, τ is the fluorescence lifetime of the fluorophore, and ϕ is the rotational correlation time for the diffusion. The rotational correlation time of a molecule is related to the molecular weight, M, of that molecule by:

$$\phi = \left(\frac{\eta M}{RT}\right)(v + h) \quad (5)$$

where η is the viscosity of the solvent, v is the specific volume of the molecule, and *h* is the hydration of the molecule. Thus the rotational correlation time and the measured anisotropy are sensitive to changes in the molecular weight of the fluorescent molecule.

Steady State Anisotropy (20, 21)

Steady state anisotropy experiments focus on the structure of the molecule as a whole. Rotational diffusion, the tumbling of a molecule in solution, alters the orientation of a molecule relative to the plane of excitation. Rotational diffusion occurs more rapidly for smaller molecules than for larger ones, and the relationship between ϕ and M (Eq 5) is proportional. Because the relationship between ϕ and r is also proportional (Eq 4), increases in the mass of a fluorescent molecule result in an increase in the measured anisotropy.

Because anisotropy is sensitive to molecular mass, steady state anisotropy measurements are particularly suited to protein-protein binding studies. A putative binding partner for a fluorophore containing protein can be titrated and the effects observed. If the two proteins interact, then the apparent mass of the fluorophore would increase and the measured anisotropy value would also increase. This provides a qualitative assay for interaction between two protein species.

Steady state anisotropy measurements can also provide a quantitative description of the interaction between two species. Assuming a one-to-one binary complex for the interaction between species A and B the dissociation constant is given by:

$$K_D = \frac{[A]_T [B]_T}{[AB]} \tag{6}$$

where $[A]_T$ is the total concentration of A, $[B]_T$ is the total concentration of B, and [AB] is the concentration of the complex. Total measured anisotropy for the interaction between A and B where A contains the fluorophore can be expressed as:

$$r = [A]_F r_F + [A]_B r_B \tag{7}$$

where $[A]_F$ is the concentration of free A, r_F is the anisotropy of free A, $[A]_B$ is the concentration of bound A, and r_B is the anisotropy of bound A. Because quantum yield of the fluorescent moiety may change upon binding of B, the equation is expanded to:

$$r = \frac{[A]_F r_F + [A]_B r_B}{[A]_F + Y[A]_B} \tag{8}$$

where Y is the ratio of the quantum yields of bound and free A. The concentration of bound A is given by:

$$[A]_B = \frac{([A]_T + [B]_T + K_D) - \sqrt{([A]_T + [B]_T + K_D)^2 - 4[A]_T[B]_T}}{2} \quad (9)$$

The value of K_D for the interaction between A and B can be calculated by fitting measured anisotropy values to these two equations. Thus steady state anisotropy can be used both as a binding assay and to quantitatively describe the interaction between two species. This technique can be applied to protein-protein or protein-DNA interactions.

Time Resolved Anisotropy Decay (20, 21)

Time resolved anisotropy decay views the motion of a molecule in more detail than does steady state anisotropy. Following pulsed excitation with plane polarized light, anisotropy decay curves are obtained over time by alternately recording emission oriented parallel and perpendicular to the plane of excitation (Eq 3). For a perfectly spherical rotator, the anisotropy decay curve is also given by:

$$r(t) = r_0 \beta e^{-t/\phi} \quad (10)$$

where ϕ is the rotational correlation time and β is its preexponential term. For a molecule which rotates anisotropically, r(t) is modeled by a sum of exponentials,

$$r(t) = r_0 \sum \beta_j e^{-t/\phi_j} \quad (11)$$

where ϕ_j is the rotational correlation time of the jth component, and β_j is its preexponential term.

For tryptophan fluorescence in proteins, anisotropy decay is typically influenced by two types of motion: the motion of the segment of protein in which the tryptophan is located and the motion of the protein as a whole. This anisotropy decay is described by the sum of two exponentials:

$$r(t) = r_0\left(\beta_1 e^{-t/\phi_1} + \beta_2 e^{-t/\phi_2}\right) \qquad (12)$$

where β_1 and ϕ_1 are the parameters which describe the segmental motion of the protein and β_2 and ϕ_2 describe the global motion of the protein.

The anisotropy decay parameters β and ϕ provide information about the structure of the protein. The preexponential terms describe the contribution of their respective rotational correlation times to the total anisotropy. This gives information as to the relative importance of the motions in the protein. Anisotropy of a molecule which is tumbling rapidly but with a rigid segment surrounding the probe, for example will be dominated by the global motion and have a higher β_2 value. The contribution of β_1 or β_2 is taken as a fraction of their sum.

As described previously, an increase in ϕ reflects an increase in the apparent molecular weight of the protein. For the global motion term, ϕ_2, changes in ϕ_2 value in the presence of a potential interaction partner for a protein can indicate the formation of a complex between the two. The

segmental motion term, ϕ_1, however does not reflect the protein as a whole, but only a portion thereof. Changes in segmental motion indicate changes in the degree of order of the segment of protein surrounding the tryptophan residue. If the limits of the segmental motion of the tryptophan are modeled as the tryptophan wobbling in a cone (17, 23), the cone semiangle, Θ, describes the extent of the segmental motion. This can be calculated from the decay parameters by:

$$\beta_2 = r_0\left[\tfrac{1}{2}(\cos\Theta)(1+\cos\Theta)^2\right] \qquad (13)$$

Changes in the contribution of ϕ_1 to the total anisotropy and in the cone semiangle in the presence of a binding partner describe the effects of the formation of a complex on the structure of the segment of the protein containing the tryptophan fluorophore. Taken together, segmental and global motion parameters provide low resolution protein structural data and information about the effects of binding interactions on that structure.

Fluorescence Spectroscopy and the Study of Transcription

Fluorescence spectroscopy can provide a considerable amount of information about protein and DNA. It can be used to study the dynamics of interactions between proteins and/or nucleic acids. As described above, anisotropy experiments can provide dissociation constants for interactions and low resolution structural information about the segmental and global motion in a protein. Fluorescence

experiments can also provide information about intermolecular distances, molecular orientation, conformational transitions induced by ligand binding or self-association, and protein folding or unfolding. The effects of changes in temperature, pH, or solvent composition on protein structure and/or interaction can be investigated. Usually these types of experiments involve a Trp label. Sometimes it is DNA which is labeled. For the purposes of this thesis, I will limit the discussion to the uses of fluorescence spectroscopy to study transcription.

Several prokaryotic transcriptional components have been studied in detail using fluorescence. Heyduk, Lee, and their colleagues have made extensive use of both native tryptophan and the 5-hydroxytryptophan analog, and covalently attached extrinsic probes in experiments with the cAMP receptor protein (CRP) of *E. coli*. Fluorescence anisotropy has demonstrated the binding of CRP to the α subunit of *E. coli* RNA Polymerase (RNAP) (9), and proven the existence of two distinct CRP-cAMP complexes, CRP-cAMP and CRP-$(cAMP)_2$ (11). Fluorescently labeled DNA oligos have also been used in anisotropy experiments to determine the affinity of these two CRP forms for operator DNA (10). Fluorescence resonance energy transfer experiments, in which non-radiative transfer of fluorescence from one fluorophore to another is used as a measure of the proximity of the two moieties, have also been employed in this system to determine the degree of CRP induced DNA bending (12). Fluorescent probes must be within a certain, probe-

specific distance for resonance energy transfer to occur. For probes placed on opposite ends of a DNA oligo, the actual distance between the probes can be calculated from experimental data and the angle of the DNA inferred.

The study of *E. coli* RNAP itself has also been amenable to fluorescence techniques. Fluorescence resonance energy transfer has been used to determine the distances between and relative orientation of positions in the β and σ subunits of RNAP (5). More recently, anisotropy techniques have been employed to study the interaction of RNAP with promoter DNA (6) and σ70 (4), and the conformational states of RNAP during the process of transcriptional initiation (32).

Fluorescence has also been used to investigate open complex formation by making use of fluorescence quenching, the damping or elimination of fluorescence intensity upon contact with some quencher molecule. The intrinsic fluorescence of the DNA base analog 2-aminopurine (2-AP) is quenched by DNA base pairing. Thus the single stranded or double stranded state of 2-AP containing DNA can be determined by the presence or absence of 2-AP fluorescence. Moreover, because the transcription bubble formation and 2-AP fluorescence lifetime are on the same time scale, transcription bubble formation can be followed in real-time using time resolved spectroscopic techniques. This technique has been used to follow the transcription bubble in RNAP transcription (6, 34) and in the study of bacteriophage T7 RNA

polymerase (31). Fluorescence quenching and anisotropy techniques have also been applied to the study of the *E. coli* trp repressor (7, 22, 29, 30), lac repressor (2), H-NS transcription factor (36), and Tet repressor (28, 37).

Recently, fluorescence spectroscopy techniques have also been applied to problems of eukaryotic transcription. Kersten, Noy, and colleagues have performed in depth fluorescence studies of the retinoid X receptor (RXR). Using steady state fluorescence anisotropy of intrinsic tryptophan probes, they have obtained K_D values for the dimerization and tetramerization of RXR (13) and the effect of mutations which disrupt transcriptional activation on these multimerizations (16). They have also applied fluorescence anisotropy to the interaction between RXR and its ligand 9-cis-retinoic acid to show that binding of the ligand causes dissociation of RXR tetramers in a positively cooperative manner (14, 15).

Fluorescence spectroscopy has also been used to study one of the central proteins in eukaryotic transcription, TBP. Experiments undertaken in this laboratory used fluorescence spectroscopy to determine that the transcriptional activation domain of the herpes simplex protein VP16 adopts a more ordered structure upon binding to TBP (33). Emission spectroscopy has been used to demonstrate an effect of DNA binding on the structure of an N-terminal portion of yTBP, although the DNA binding domain of yTBP is located in the C-terminal

portion of the protein (27). Anisotropy studies have been used to calculate a K_D for the interaction of TBP and TATA oligo, and to show that in the absence of DNA, TBP forms a multimer in solution (27). Parkhurst, et al. have used resonance energy transfer to calculate the DNA bend induced by TBP in solution for comparison with the results of X-ray crystallographic analysis (25). Most recently, fluorescence anisotropy has been used to study the interaction of TBP and $yTAF_{II}130$. A K_D for this interaction has been calculated, and it has been shown that the N-terminus of this protein can dissociate TBP from TATA box DNA (1). Fluorescence techniques have also been used to test the sequence specificity of DNA binding by TFIIB (19), determine the degree of DNA induced bending by a fission yeast transcriptional regulator (24), observe effects of mutations in c-Myb on its conformation (8), observe effects of binding of DNA by Fos and Jun on protein conformation (26), and study the effects of transcription by PolII on nucleosome structure (3).

Clearly there is enormous evidence of the utility of fluorescence spectroscopy in the study of protein-DNA and protein-protein interactions. Fluorescence experiments require only limited (nM) quantities of protein and are performed in solution, conditions which allow fluorescence spectroscopy to more closely mirror physiological conditions than other methods used to study protein structure and interactions (20, 21). In experiments described later in this thesis, we make use of fluorescence emission and anisotropy to investigate in more

detail the interactions of the transcriptional activator VP16 with TBP. Further applications of fluorescence to the study of VP16 and transcription are proposed in the conclusion of this thesis.

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CHAPTER 3

MUTATIONAL ANALYSIS OF A TRANSCRIPTIONAL ACTIVATION DOMAIN OF THE VP16 PROTEIN OF HERPES SIMPLEX VIRUS*

INTRODUCTION

Basal levels of transcription of eukaryotic mRNA genes by RNA polymerase II can be augmented by the action of proteins known as transcriptional activators. Several models have been proposed for the function of eukaryotic activators, including the recruitment of chromatin remodeling enzymes (22) or of basal transcription factors. These latter interactions might facilitate pre-initiation complex formation (40), increase initiation rate or aid promoter clearance (52), or increase the rate of elongation (4). Each of these models invokes specific interactions between transcriptional activation domains and particular target proteins that are likely to require certain structural motifs present in the activator protein. Therefore, transcriptional activators have been studied intensively to identify such activation motifs.

Eukaryotic transcriptional activators were initially described by the amino acids most abundantly present, resulting in classes of acidic, glutamine-rich, proline-rich, and serine/threonine-rich domains (34,

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*The work described in this chapter is currently in press:
Sullivan, S.M., P.J.Horn, V.A. Olson, A.H.Koop, W.Niu, R.H.Ebright, and S.J.Triezenberg. 1998. Mutational analysis of a transcriptional activation region of the VP16 protein of herpes simplex virus. Nucleic Acids Res.

52), although some activation domains do not fit this scheme (2). Thorough mutational analyses of acidic activation domains from VP16 (9, 42), RelA (6), p53 (31), Bell (28), GCN4 (20), the glucocorticoid receptor (1, 19), C1 (45), and of a glutamine rich activation domain from Sp1 (13), indicated that the most prevalent types of amino acid were not those most critical for activation function. Rather, in each case, specific bulky hydrophobic or aromatic amino acids were more important.

This conclusion from mutational analyses is consistent with results from biophysical experiments. Although activation domains, in isolation, typically appear highly unstructured (11, 38, 46, 48, 55), more ordered structures can be induced by the presence of putative target proteins. Among the examples reported to date are the interaction of the VP16 activation domain with TBP (49) and with hTAF$_{II}$32 (54), the interaction of the p53 transcriptional activation domain with its repressor MDM2 (27), and the interaction of an activation domain from CREB with a fragment of CBP (41). In each case, the interaction surface of the activator comprised primarily hydrophobic residues (including many defined by mutational analysis) within helical segments, with a notable lack of participation by acidic sidechains.

VP16 (also known as Vmw65 or α-TIF) is a component of the virion of herpes simplex virus whose transcriptional role is to activate expression of the viral immediate early genes (reviewed in 37). The 490 amino acids of VP16 can be divided into two domains by biochemical or

molecular genetic methods (17, 53). A domain comprising the amino terminal 410 amino acids forms a complex with the host proteins Oct-1 and HCF that can bind to specific DNA sequences in the promoters of the immediate early genes (37). The carboxyl terminal 80 amino acids of VP16 are both necessary and sufficient for transcriptional activation, even when fused to a heterologous DNA-binding domain (8, 44, 53). The VP16 activation domain can be further divided into two subdomains herein termed VP16N (amino acids 412 to 450) and VP16C (amino acids 450 to 490), each of which is independently capable of activation when provided with a DNA binding mechanism (15, 42, 47, 53, 56).

Extensive mutational analysis of the VP16N subdomain indicated a key role for the aromatic character of F442 and supporting roles for other bulky hydrophobic residues, specifically L439 and L444 (9, 42). The acidic residues in this domain had a much less important role. Any one or even several negative charges could be removed without significant loss of function. We and others have previously tested the roles of several amino acids within the carboxyl-terminal subdomain of VP16. In one case, several substitutions of F473 and F475 were tested in the context of full-length VP16 bearing the debilitating F442A mutation in the N subdomain, with the result that mutations at F475 had a somewhat greater effect than corresponding mutations at F473 (42). In another study, the simultaneous mutation of all three phenylalanine residues within the C subdomain (i.e., F473, F475, and F479) to alanine had a

dramatic effect on the ability of that subdomain to function in the context of the full-length activation domain (56).

These preliminary analyses, however, are insufficient to ensure that all key amino acids in VP16C have been identified, and fall short of defining the chemical characteristics of those amino acid sidechains that contribute to the transcriptional activity of this domain. To comprehensively identify the critical residues in VP16C and to further test the hypothesis that specific patterns of hydrophobic residues are important for function of activation domains, we have undertaken a thorough mutational analysis of VP16C using both PCR-mediated random mutagenesis and systematic alanine-scanning mutagenesis. Random mutagenesis permits the analysis of a broad range of amino acid substitutions altering the size, charge and hydrophobicity of the amino acid sidechains. Moreover, the approach as applied here has the advantage of an *in vivo* screening step which allows selection of mutants defective in transcriptional activation. The complementary strategy of alanine-scanning mutagenesis ensures that each amino acid position will be tested and indicates the contributions of the side-chain atoms of a given residue, because alanine substitutions eliminate all sidechain atoms beyond the β-carbon (10) . The combination of these complementary mutational analyses, therefore, provides information about each position while focusing on those which affect activation most strongly. The results indicate that in the case of VP16C, as with other

activation domains, specific hydrophobic amino acids are particularly critical to its transcriptional function.

MATERIALS AND METHODS

Plasmids and Phage. The substrate for all mutagenesis reactions was mpPJH18, an M13mp19 phagemid containing a *Sma* I to *Bam*H I fragment encoding VP16 residues 452-490 with 119 bp of 3' untranslated sequence. Mutants generated by error-prone PCR were screened in yeast using pPJH13, a high copy (2μ *LEU2*) plasmid that expresses a Gal4(1-147)-VP16C fusion protein from the *ADH1* promoter. Quantitative assays of transcriptional activation in yeast were performed using wild type or mutant versions of pVS1, a low copy plasmid (*CEN6 ARSH4 LEU2*) that expresses the Gal4(1-147)-VP16C fusion protein, and the yeast reporter construct pLGSD5 (16) containing the *E. coli lacZ* structural gene with the *GAL1-10* upstream activating sequence near the *CYC1* promoter. VP16C mutants were also assayed in mammalian cells using pSM71-1.VP16C, a derivative of pSGVP (44) that expresses the Gal4(1-147)-VP16C fusion protein from the SV40 early-gene promoter. The mammalian reporter plasmid pG_5B_{CAT} (30) expresses the bacterial chloramphenicol acetyltransferase protein under the control of five Gal4 sites upstream of the TATA sequence from the adenovirus E1B promoter.

Error-prone PCR-Meditated Mutagenesis. To generate random nucleotide substitutions, the VP16C coding domain of mpPJH18 was

amplified by PCR using forward and reverse M13 sequencing primers under reaction conditions that diminish the accuracy of *Taq* DNA polymerase, including the presence of $MnCl_2$ (29) and limiting concentrations of dATP (50). Reaction mixtures included 10 mM Tris-Cl (pH 8.8), 50 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.4 mM dATP, 3.2 mM each dCTP, dGTP, dTTP, 100 μg/ml gelatin, 10 ng of mpPJH18, 60 pmol of each primer, and 5 U of Taq DNA polymerase (Perkin-Elmer) in a final volume of 100 ul. After an initial melting step of 2 minutes at 94° C, the temperature was lowered to 80° C and PCR was initiated by the addition of nucleotides and polymerase. Amplification proceeded through 25 cycles (30 sec. annealing at 43° C, 2 min. elongation at 72° C, 30 sec. denaturation at 94° C). The PCR products were digested with *Sal*I and *Bam*H I, ligated into pPJH13 and transformed into yeast strain BP1 [*MAT*a *ura3-52 leu2-3, 2-112 Gal4::HIS4 ade1-100*; , ref. (5)].

Alanine Scanning and Site-directed Mutagenesis. Oligonucleotide-directed mutations were generated in mpPJH18 using the method described by Kunkel et al. (26). In some cases, a variety of substitutions at specific positions were generated using degenerate oligonucleotides. *Sal* I to *Bgl* II fragments containing the mutations were subcloned into pVS1 and resequenced to verify the identities of mutations.

Yeast Transformation and β-galactosidase Assay. Plasmids expressing VP16C mutants fused to the Gal4 DNA-binding domain were co-transformed with the reporter plasmid pLGSD5 into yeast strains

BP1or PSY316 [*MATa ade2-101 his3-del.200 leu2-3,2-112 lys2 ura3-53*, ref. (5)] using the procedure of Gietz *et al.* (12). To assay the activity of β-galactosidase produced from pLGSD5, 2 ml cultures containing pools of approximately 100 yeast transformant colonies were grown to early stationary phase (OD600 = 0.9-1.2) in selective media, then diluted to OD600 = 0.1, grown to mid-log phase (OD600 = 0.5) and harvested by centrifugation. β-galactosidase activity was measured (43) using extracts obtained from 5 mL cultures with results normalized to protein concentration as determined by Bradford assay (7).

Yeast Immunoblot Analysis. To assess the steady-state levels of each mutant activator protein, 25 ml yeast cultures grown as described above were harvested by centrifugation, washed once in water, and resuspended in water to a total volume of 100 µl. An equal volume of 4x SDS-PAGE loading buffer lacking bromphenol blue was added and extracts were boiled for 15 minutes, then centrifuged for 5 minutes at 14,000 *g* to clarify the extract. Relative protein concentrations for each extract were determined using a detergent compatible protein assay (Bio-Rad), and approximately 10 µg total protein was loaded per sample onto 15% SDS-PAGE gels. Following electrophoresis, proteins were electrophoretically transferred to nitrocellulose. Membranes were blocked in 10% powdered milk, 20 mM TrisCl pH 7.5, 137 mM NaCl, 4mM KCl, 0.01% Tween 20 and then incubated sequentially with rabbit polyclonal

antibodies raised against the Gal4-VP16 fusion protein (LA2-3; Lee Alexander and Steven J. Triezenberg, unpublished) followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad). Blots were developed using an enhanced chemiluminescence system (ECL, Amersham or Renaissance, DuPont NEN Life Science Products).

Mammalian Plasmid Construction and Assay. The VP16C mutant activation domains were subcloned from the yeast plasmids into pSM71-1.VP16C. All subclones were sequenced to verify their identities. 100 ng of each plasmid was cotransfected into mouse L cells (American Type Culture Collection) with 1 μg pG_5B_{CAT} using DEAE-dextran (33). Plates were incubated at 37°C, 10% CO_2 for 36-48 hours after which cells were harvested by scraping into phosphate-buffered saline followed by centrifugation. Cell extracts were made by freeze-thawing and CAT assays were performed by the mixed-phase method (35, 36). Final reaction volumes of 100 μl contained 30 μl of cell extract, 200 mM Tris-Cl pH7.8, 4 mM EDTA, 30 mM acetyl-CoA, and 0.4 μCi [^{3}H]-acetyl CoA. Reactions were overlaid with 10 ml Econofluor2 (Packard Industries), incubated for 2 hours, and quantitated using an LKB Wallace 1209 Rackbeta liquid scintillation counter. Counts were normalized to protein concentration as determined by Bradford assay (7).

Mammalian Western Blot Analysis. Separate plates of L cells were transformed with 5 μg of each plasmid encoding wild type or mutant

Gal4-VP16 fusion protein in the manner described above. After 36-48 hours, cells were washed once in phosphate-buffered saline and harvested by scraping into 200 µl of 4xSDS-PAGE loading buffer lacking bromphenol blue. Samples were boiled for 10 minutes and then spun for 5 minutes at 14,000 *g* to clarify the extracts. Relative protein concentrations were determined by using the BioRad detergent-compatible protein assay and Western blots were performed as described above.

RESULTS

Biological selection of transcriptionally defective mutants of VP16C

Expression of the wild type Gal4-VP16C fusion protein from a high-copy plasmid is relatively toxic to yeast, as is the fusion protein bearing the full-length VP16 activation domain (5). As a result, yeast strains expressing high levels of these activators produce very small colonies. Mutations in the VP16 activation domain that reduce transcriptional activation also reduce the toxicity (5), yielding larger yeast colonies. This phenomenon was employed to screen a library of VP16C clones to identify mutations that negatively affect transcriptional activity. A DNA fragment encoding the VP16C subdomain was amplified using *Taq* DNA polymerase under error-prone conditions. The population of amplified fragments was then ligated into a yeast expression vector such that the VP16 coding sequences were in-frame with sequences encoding

the Gal4 DNA-binding domain. This library was then transformed into yeast cells and large colonies were selected. DNA fragments encoding the VP16C subdomain were isolated from these colonies and were sequenced. These fragments were then cloned into a low-copy yeast plasmid from which expression of the Gal4-VP16C fusion protein is sufficiently low to avoid the toxicity. Transcriptional activation by the mutant fusion proteins was quantitatively assessed using a β-galactosidase reporter gene with a Gal4-responsive promoter (Figure 1).

Of the fourteen PCR-generated mutants in VP16C isolated by this approach, eleven have at least one amino acid substitution between positions 470 and 480. Eight of the thirteen missense mutants have changes at one or more of the phenylalanines in this domain (F473, F475, and F479). Moreover, all mutants displaying 40% or less of wild type activity bear a substitution at one or more of these phenylalanine residues, with the exception of the L438P/M478V mutant. For two of these three phenylalanines, substitution to leucine resulted in only a relatively modest defect: in β-galactosidase assays, F475L and F479L displayed 78% and 90% of wild type activity, respectively. Substitutions at these positions to a hydrophilic residue such as serine, however, resulted in large defects. The mutant F473S retained only 26% of wild type activity, and the two mutants with serine substitutions at F479 (F479S/D486G and F475S/M478T/F479S), showed 10% or less of wild type levels. These results indicate the important role of bulky

Figure 1. Mutation identities and relative activities of VP16C mutants selected for reduced toxicity when highly expressed in yeast. The Gal4-VP16C fusion protein, when expressed from the *ADH1* promoter on a high-copy plasmid, is toxic to yeast resulting in tiny colonies. The VP16C mutants listed here, generated by error-prone PCR, were selected based on larger yeast colony size. The VP16C subdomains were then recloned into a low-copy vector (to avoid toxicity) and assayed for transcriptional activity using a Gal4-reponsive *lacZ* reporter plasmid (pLGSD5). Below the amino acid sequence of VP16C (residues 450-490) are shown the positions and types of substitutions identified in each of these mutants. The β-galactosidase activities in lysates of cells containing each of the Gal4-VP16C mutants are expressed relative to the activity of the wildtype Gal4-VP16C fusion protein.

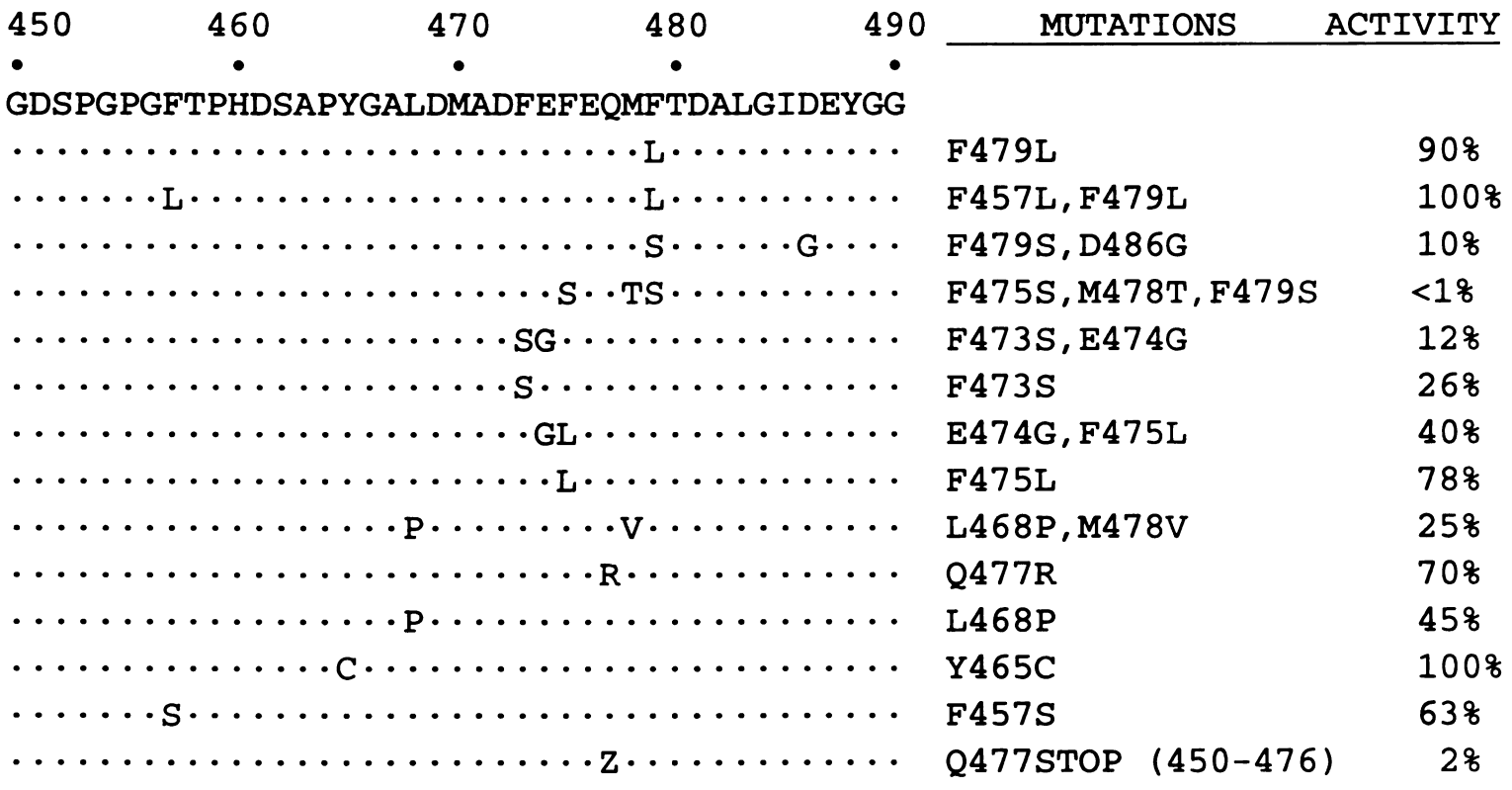
450 460 470 480 490
MUTATIONS ACTIVITY
GDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG
F479L 90%
F457L,F479L 100%
F479S,D486G 10%
F475S,M478T,F479S <1%
F473S,E474G 12%
F473S 26%
E474G,F475L 40%
F475L 78%
L468P,M478V 25%
Q477R 70%
L468P 45%
Y465C 100%
F457S 63%
Q477STOP (450-476) 2%

Figure 1

hydrophobic or aromatic groups at positions 473, 475, and 479 for transcriptional activation by VP16C.

Interestingly, the fourth phenylalanine residue in VP16C, F457, seems much less important. This position was altered in only two of the mutants isolated by this approach. The F457L/F479L mutant activated the reporter gene at nearly wild type levels. The F457S mutant showed 63% of wild type activity, a modest reduction compared to serine substitutions at the other phenylalanine positions. This result supports the idea that residues between positions 470 and 480, not simply any bulky hydrophobic or aromatic residues in the vicinity, were primarily responsible for the transcriptional activity of VP16C.

Alanine scanning mutagenesis of VP16C

As a complement to the random mutagenesis and to more systematically assess the importance of each residue of VP16C individually, we constructed alanine substitutions at each position (except those that were originally alanine or glycine). The 27 mutants created in this set were tested as Gal4-VP16C fusion proteins, expressed from a low-copy plasmid, for the ability to activate a Gal4-responsive □-galactosidase reporter gene in yeast. The results (Figure 2) support the conclusion derived from the PCR-generated mutants that the most critical residues for activation by VP16C lie between positions 470 and 480. Only four of the twenty-seven alanine mutants have activity less than 70% that of wild type. Three of these mutations affect

Figure 2. Relative activities in yeast of alanine-scanning mutants of the VP16C subdomain. Alanine substitutions were constructed at each position of VP16C except glycines and existing alanines. The activities of Gal4-VP16C fusion proteins bearing these substitutions were assayed using a β-galactosidase reporter gene as described in the legend to Figure 1. The position of each mutation is represented by the amino acid sequence of VP16C along the horizontal axis of this figure. Bars indicate mean activity (with standard deviation) of β-galactosidase in yeast cell extracts from at least three parallel cultures, expressed relative to the activity of the wildtype Gal4-VP16C fusion protein (indicated by +). β-galactosidase activities in extracts lacking the Gal4-VP16C fusion protein (indicated by -) were negligible. A double substitution of alanine for both F473 and F475 is shown at the right end of the figure.

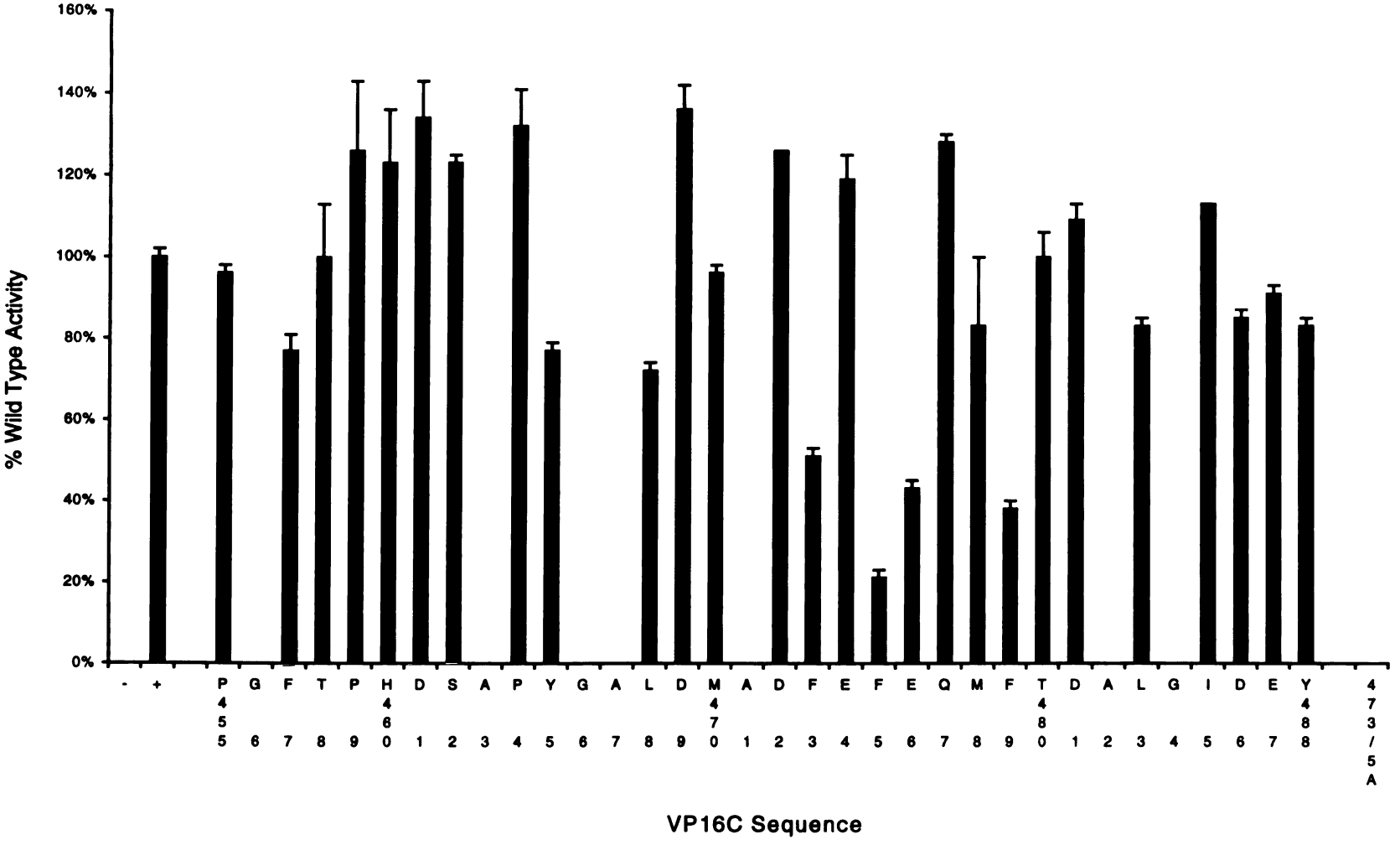
% Wild Type Activity
160%
140%
120%
100%
80%
60%
40%
20%
0%
- + P455 G6 F7 T8 P9 H460 D1 S2 A3 P4 Y5 G6 A7 L8 D9 M470 A1 D2 F3 E4 F5 E6 Q7 M8 F9 T480 D1 A2 L3 G4 I5 D6 E7 Y488 473/5A
VP16C Sequence

Figure 2.

phenylalanines: F473A (51% activity), F475A (21%), and F479A (43%). A double mutant with alanine substitutions at both positions 473 and 475 showed 1% or less activity. The fourth mutant with a significant loss of activity was E476A, which displayed only 38% of wild type activity. This result suggests that the sidechain of E476 contributes to the function of VP16C, despite the fact that this site was not affected in any of the randomly-generated mutants isolated in the previous experiment. All other alanine substitutions had little or no effect on transcriptional activation in yeast assays.

To determine whether activation by VP16C in mammalian cells depends on the same amino acids as in yeast, the alanine substitution mutants were also assayed as Gal4 fusion proteins in mouse L cells. Plasmids expressing the Gal4-VP16C proteins from the SV40 early promoter were cotransfected with pG_5B_{CAT}, a reporter plasmid in which expression of chloramphenicol acetyltransferase (CAT) is controlled by Gal4-binding sites (30). After 36-48 hours, the CAT enzyme activity in cell extracts was assayed as an indication of the ability of the Gal4-VP16C protein to activate transcription. The results shown in Figure 3 are very similar to the results of assaying the mutants in yeast. As in yeast, the mutants with the most significant effects (i.e., activity less than or equal to 60%) have substitutions of the phenylalanine residues at positions 473 (49%), 475 (25%), or 479 (22%), or the glutamate residue at 476 (60%). Two other mutants, T458A and H460A, have

Figure 3. Relative activities of Gal4-VP16C alanine-scanning mutants in mammalian cells. Each of the alanine-scanning mutations described in Figure 2 was recloned into a mammalian expression plasmid and transfected into mouse L cells with a reporter gene that expresses chloramphenicol acetyltransferase under control of Gal4 binding sites (pG5Bcat). The CAT enzyme activities in cell extracts were assayed by the fluor-diffusion method, adjusted to ensure that measurements were within the linear range of the assay. Bars indicate mean activities (with standard deviations) of CAT activity from at least three plates of cells transfected with a given Gal4-VP16C mutant expression plasmid, relative to the activity of the wildtype Gal4-VP16C plasmid (indicated by +).

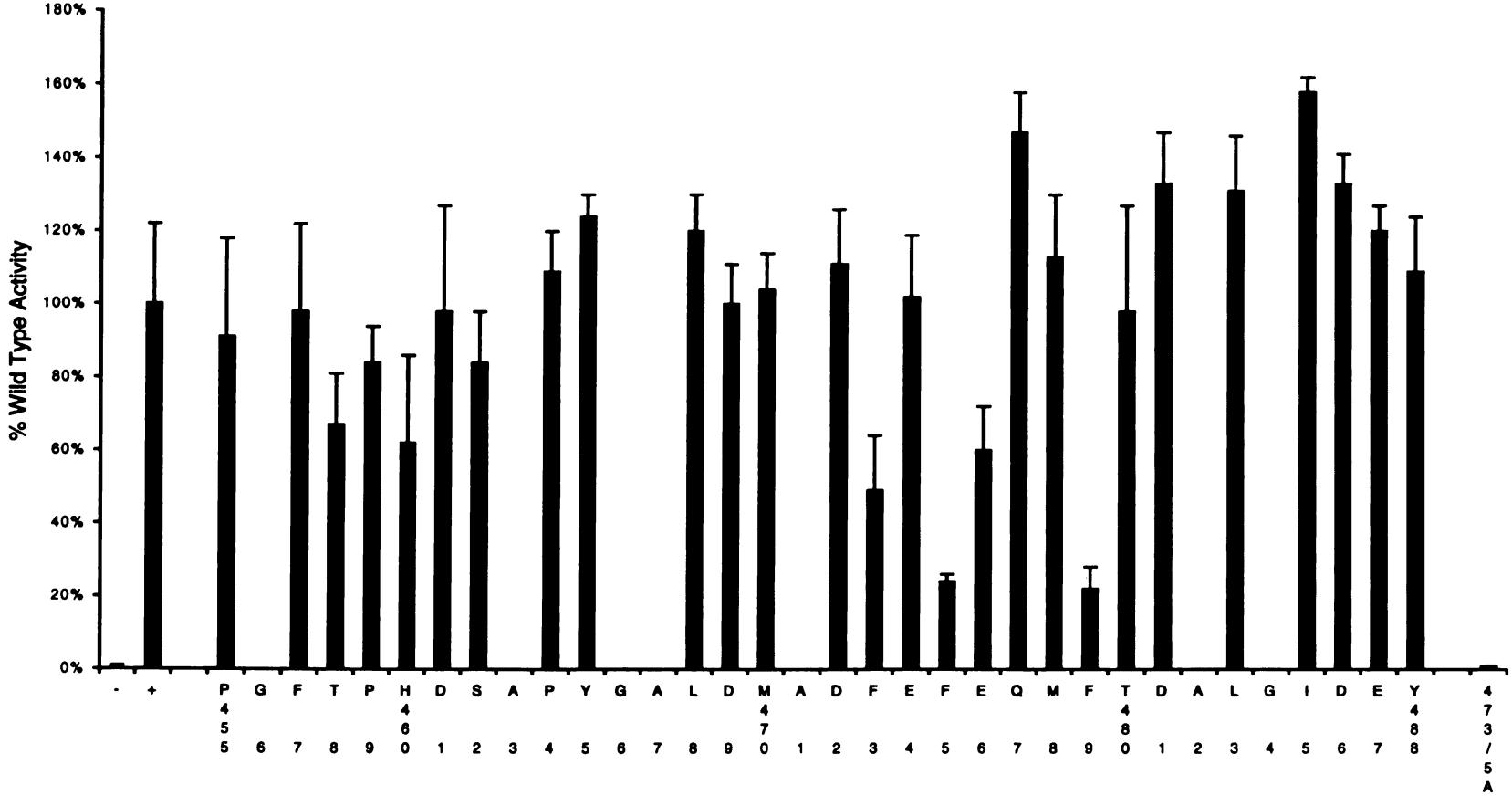
% Wild Type Activity
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
- + P455 G6 F7 T8 P9 H460 D1 S2 A3 P4 Y5 G6 A7 L8 D9 M470 A1 D2 F3 E4 F5 E6 Q7 M8 F9 T480 D1 A2 L3 G4 I5 D6 E7 Y488 473/5A
VP16C Sequence

Figure 3.

activities less than 70% (67% and 62%, respectively) in mammalian cells, although their activities in yeast assays were close to wildtype levels. All other alanine substitutions had little or no effect on transcriptional activity in mammalian cells.

Side-chain preferences at key positions in VP16C

The random and systematic mutational analyses described in the preceding sections indicate that F473, F475, E476 and F479 are the residues most critical for the transcriptional function of VP16C in both yeast and mammalian systems. The strong activity of the mutants bearing phenylalanine to leucine substitutions, derived from the PCR-mediated mutagenesis, suggested that the hydrophobic character of the amino acids at those positions is more important than is the aromatic character, in contrast to previous observations for the F442 residue in VP16N. Therefore, to more clearly determine the chemical characteristics most important for the sidechains of the amino acids at these positions, we constructed a number of substitutions at each site and tested the ability of these mutants to activate transcription in yeast and in mammalian cells.

Each of the three key phenylalanine residues was altered to other aromatic amino acids, non-aromatic bulky hydrophobic amino acids, small hydrophobic amino acids, small hydrophilic residues, and charged or polar residues. When assayed in yeast, the effects of a particular type of substitution were quite similar at each of the three Phe positions

tested (Figure 4A, 4B, 4E). Substitutions of phenylalanine with either aromatic or bulky hydrophobic residues resulted in proteins with at least 50% of wild type activation ability. Even a double mutation to leucine at positions 473 and 475 retained approximately 60% of wild type activity (Figure 4B). Alterations at these positions to residues with smaller or hydrophilic sidechains, however, reduced activity to 50% or less. These data support the hypothesis that hydrophobic character is the most significant criterion of these residues that are critical for activation.

Interestingly, effects at positions 475 and 479 were more severe than those at 473. Changes resulting in sidechains that are not bulky and hydrophobic only reduced activity to between 50% and 30% for 473, whereas for 475 and 479, activity was less than 25% for all of these changes. This result may indicate that F473 is less important for transcriptional activation than are F475 and F479.

When assayed in mammalian cells, the substitutions at positions 475 and 479 produced results similar to those obtained in yeast. At these positions, the activities of mutants with hydrophobic amino acid substitutions were at least 60% of wild type respectively, while substitutions with non-hydrophobic residues yielded activities less than 30% that of the wild type protein (Figure 5B, 5E). Again, these results support the hypothesis that the hydrophobic nature of residues at these positions is important for activation by VP16C. A double mutant with

Figure 4. Relative activities in yeast of Gal4-VP16C mutants bearing various amino acid substitutions at specific positions. Mutant Gal4-VP16C proteins with substitutions at F473 (panel A), F475 (panel B), E476 (panel C), M478 (panel D), or F479 (panel E), were assayed for their ability to activate expression of a β-galactosidase reporter gene as described in Figure 1. The individual substitutions are indicated along the horizontal axis of each panel in descending order of activity from left to right, with the mean activity (with standard deviations) from at least three cultures for each mutant being represented by a vertical bar. Panel B also indicates the activity of one mutant with leucine substitutions at both F473 and F475.

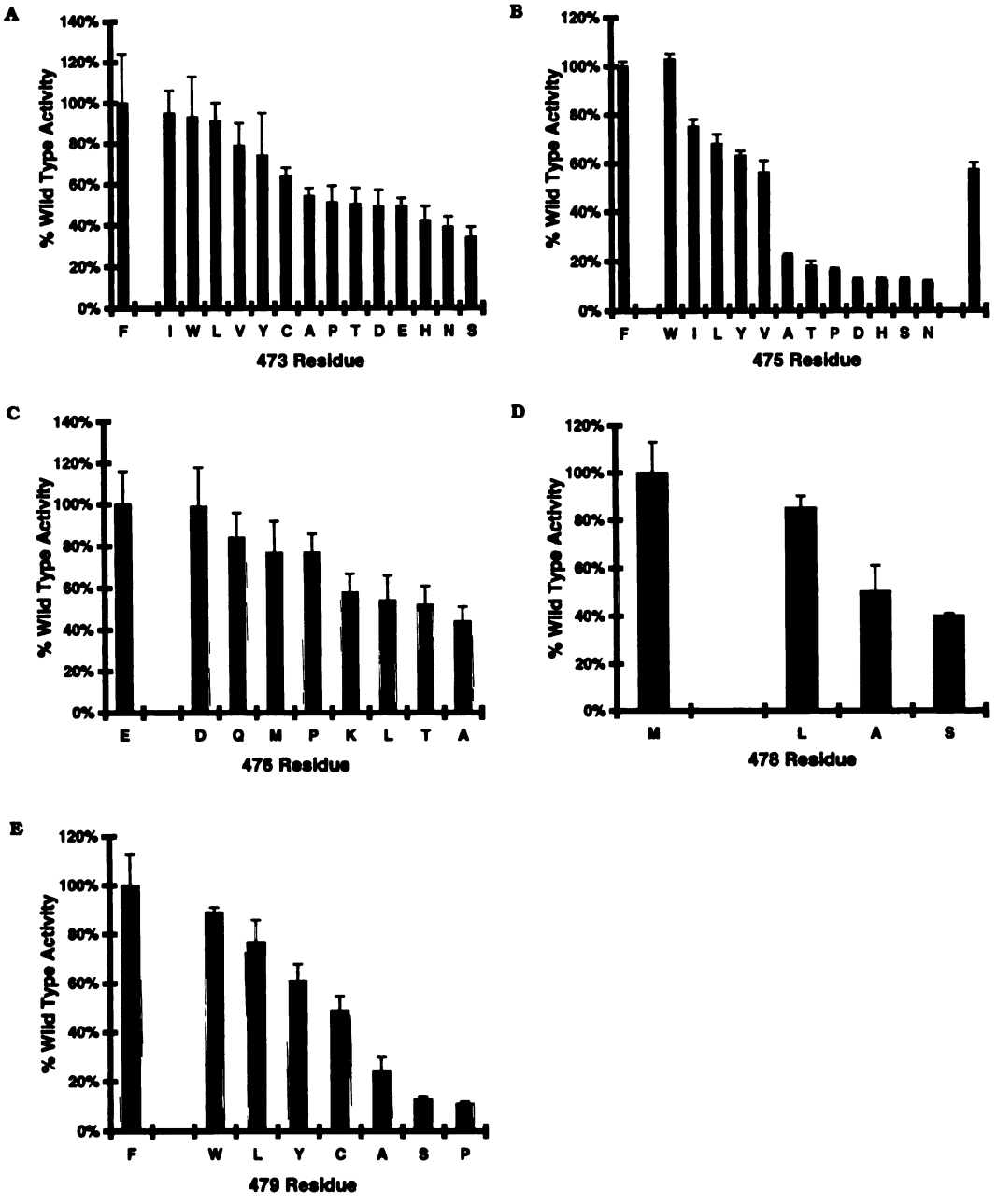
A
% Wild Type Activity
140%
120%
100%
80%
60%
40%
20%
0%
F I W L V Y C A P T D E H N S
473 Residue
B
% Wild Type Activity
120%
100%
80%
60%
40%
20%
0%
F W I L Y V A T P D H S N
475 Residue
C
% Wild Type Activity
140%
120%
100%
80%
60%
40%
20%
0%
E D Q M P K L T A
476 Residue
D
% Wild Type Activity
120%
100%
80%
60%
40%
20%
0%
M L A S
478 Residue
E
% Wild Type Activity
120%
100%
80%
60%
40%
20%
0%
F W L Y C A S P
479 Residue

Figure 4.

Figure 5. Relative activities in mammalian cells of Gal4-VP16C fusion proteins with various substitutions at specific positions. Each of the VP16C mutants described in Figure 4 was recloned into a mammalian expression vector and assayed for the ability to activate expression of a CAT reporter gene as described in Fig. 2. The individual substitutions are indicated along the horizontal axis of each panel in descending order of activity from left to right, with the mean activity (with standard deviations) from at least three plates of transfected cells for each mutant being represented by a vertical bar.

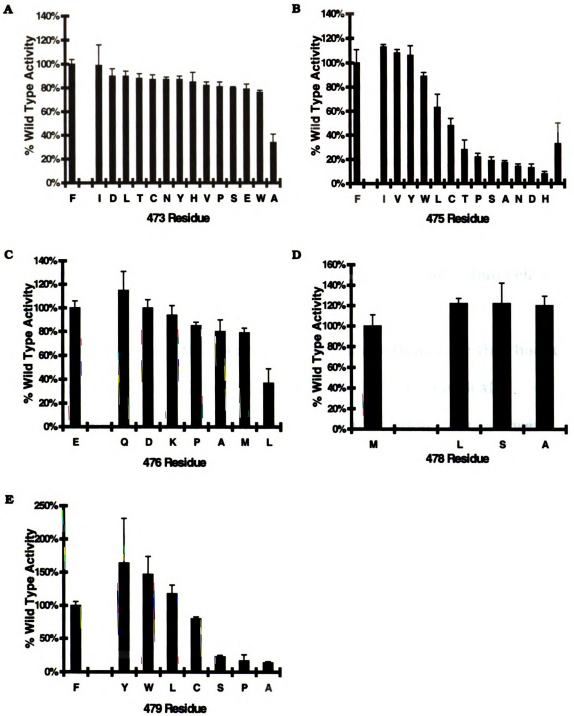
A
% Wild Type Activity
140%
120%
100%
80%
60%
40%
20%
0%
F I D L T C N Y H V P S E W A
473 Residue
B
% Wild Type Activity
F I V Y W L C T P S A N D H
475 Residue
C
% Wild Type Activity
E Q D K P A M L
476 Residue
D
% Wild Type Activity
160%
M L S A
478 Residue
E
% Wild Type Activity
250%
200%
150%
100%
50%
0%
F Y W L C S P A
479 Residue

Figure 5.

leucines at positions 473 and 475 also retained more than 30% wild type activity (Figure 5B).

The indication that F473 is less important than F475 or F479 is demonstrated even more clearly in the mammalian system than in yeast. In the mammalian assays, the only F473 mutant with activity below 75% bore a substitution to alanine (Figure 5A). Thus whereas F473 is less important in yeast than are the other phenylalanine residues, it contributes very little to the activity of VP16C in the mammalian cell assay.

The only substitution of an acidic residue with alanine that had a significant effect was E476A (Figures 2, 3). To test the chemical characteristics that might be important at that position, mutants were constructed in which the glutamate was replaced with other acidic, uncharged, nonpolar or even basic amino acids (Figure 4C, 5C). The E476D and E476Q mutants functioned with essentially wild type activity in both yeast and mammalian cells, indicating that the charge and length of the sidechain are not critical parameters. Moreover, a mutant with reversed charge (E476K) and mutants with aliphatic rather than polar sidechains (E476M, E476L, and E476P) were also at least 50% active in yeast and (with the exception of E476L) also in mammalian cells. The wide range of substitutions that do not significantly affect activity in either assay system suggests that the nature of the side chain at position 476 is not particularly critical for activation by VP16C.

Mutations at position 478 seem to affect the function of VP16C differently in the yeast and mammalian systems. In the yeast assays, leucine was an effective substitute for methionine at this position, retaining 85% activity, while alanine and serine diminished activity to 50% and 40% of wild type levels respectively (Figure 4D). In the mammalian assays, however, none of these changes significantly affected activation ability (Figure 5D). This observation suggests at least some difference in the structures required for activation in the yeast and mammalian systems.

Immunoblot Assays

The steady-state levels of all Gal4-VP16C proteins studied in this work were assessed by immunoblot analysis. Expression of Gal4-VP16 mutants in yeast cells was assayed using aliquots of the whole-cell extracts used for B-galactosidase assays. Expression of mutant fusion proteins in mammalian cells was tested by transfecting mouse L cells with five micrograms of each expression plasmid and collecting whole cell extracts two days after transfection. Samples containing equivalent amounts of total protein were electrophoresed in SDS polyacrylamide gels, blotted to nitrocellulose, and probed with a polyclonal antiserum raised against recombinant Gal4-VP16 fusion protein or a peptide representing a portion of the VP16 activation domain. The results of typical immunoblots are shown in Figure 6, which explicitly demonstrates that the mutant proteins with the greatest defects in

Figure 6. Immunoblot analysis of yeast (panel A) and mouse cells (panel B) expressing wildtype or mutant forms of the Gal4-VP16 fusion protein. Aliquots of whole cell extracts were electrophoresed in SDS polyacrylamide gels and blotted to nitrocellulose. The filters were incubated with polyclonal serum directed against the Gal4-VP16 protein and developed using enhanced chemiluminescence. Extracts from cells transformed with vector only are represented in the left lane of each panel. This representative figure shows the expression levels of mutant proteins with the greatest defects in transcriptional activation. Similar results were obtained for all VP16 mutants tested in both yeast and mammalian cells.

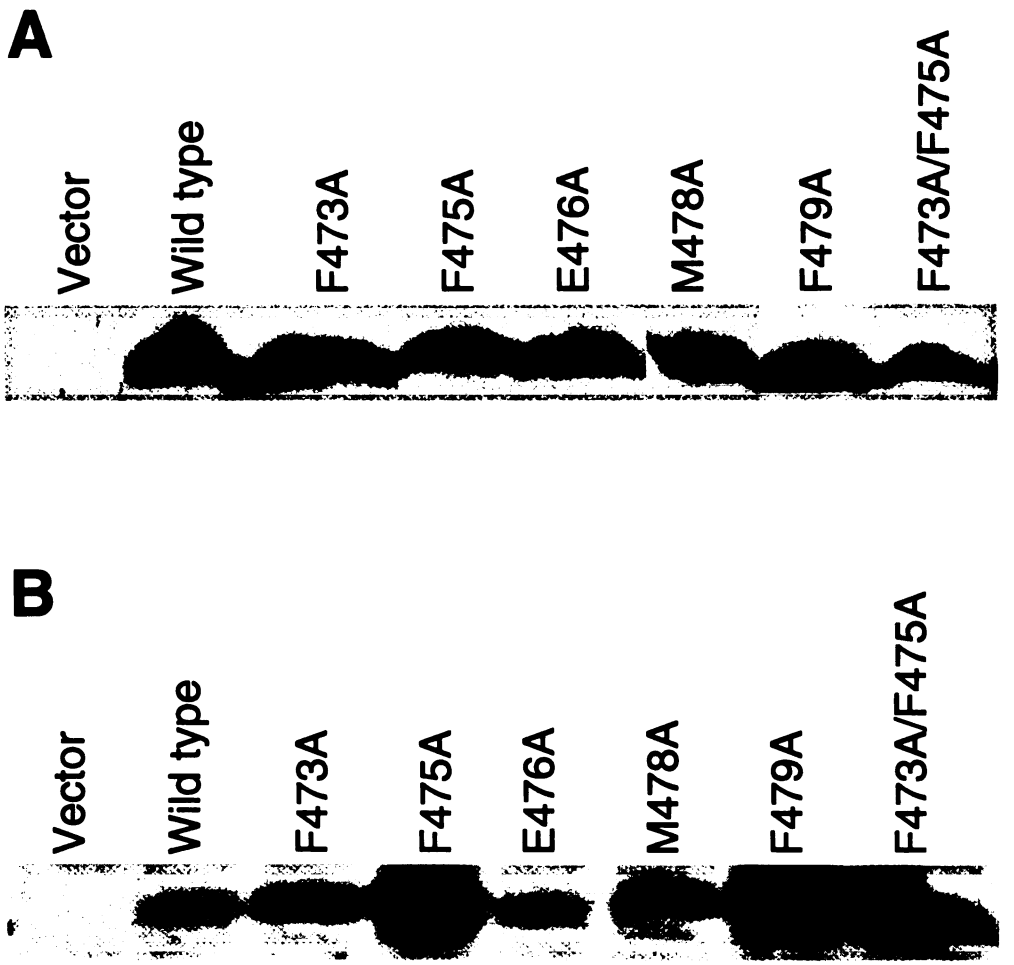
A
Vector
Wild type
F473A
F475A
E476A
M478A
F479A
F473A/F475A
B
Vector
Wild type
F473A
F475A
E476A
M478A
F479A
F473A/F475A

Figure 6.

transcriptional activity are nonetheless present at levels similar to or greater than the wildtype fusion protein. Similar results were obtained for each of the proteins whose transcriptional activities are represented in Figures 2-5 (data not shown). Therefore, the differences in transcriptional activity cannot be attributed to differences in expression or stability of the mutant activator proteins.

DISCUSSION

The first evidence that bulky hydrophobic amino acids, rather than the abundant acidic amino acids, were most critical for the function of an acidic transcriptional activation domain was observed in studies of the N-subdomain of the VP16 transcriptional activation domain [VP16N, amino acids 411-456; refs. (9, 42). In the present study, we employed two complementary mutational strategies, unbiased with respect to this hypothesis, to identify key amino acids in the VP16 C-subdomain (VP16C, amino acids 450-490). The results of both alanine scanning and PCR-mediated random mutagenesis indicated that three phenylalanine residues, at positions 473, 475, and 479, contribute significantly to activation, with F475 being the most critical. Other aromatic or bulky hydrophobic amino acid sidechains apparently contribute relatively little to transcriptional activation by VP16C, because most substitutions replacing Phe457, Tyr465, Leu468, Met470, Ile485, and Tyr 488 had little effect on transcriptional activity. We cannot exclude the possibility

that amino acid substitutions with more drastic changes in sidechain characteristics might affect transcriptional activity, as suggested by the 60% loss of activity in yeast when Leu468 was replaced by proline (Figure 1) or when Met478 was altered to serine (Figure 4D).

Thus, as previously observed for VP16N (9, 42), aromatic residues are particularly important for the function of the C-subdomain. However, the changes in chemical characteristics of those amino acid sidechains that can be tolerated with little loss of activity differ between the two subdomains of VP16. The critical phenylalanine residue of VP16N, F442, could only be functionally replaced by other aromatic residues (42). In contrast, any of the bulky hydrophobic amino acids leucine, isoleucine and valine, as well as cysteine and the aromatic residues tyrosine and tryptophan, could effectively substitute for the key phenylalanines in VP16C (Figures 4 and 5).

Although most of the acidic residues of VP16C could be altered (at least to alanine) without affecting transcriptional activation, our results indicate contributions by the glutamate residues at position 476 and possibly at position 474, two sites flanking the most critical phenylalanine. The alanine scanning results shown in Figures 2 and 3 demonstrate that the single replacement of E476 with alanine reduced the activity of VP16C by half in both yeast and mammalian cells. The negative charge of the amino acid sidechain at this position seems dispensable however, because mutants bearing nonpolar, polar, and

even basic amino acids at that position retained significant activity (Figure 4C and 5C). No other replacement of an acidic residue with alanine showed any significant effect in our experiments. However, the replacement of E474 with glycine had modest but reproducible effects in the context of the E474G/F475L and F473S/E474G double mutants when compared with the F475L and F473S single mutants (Figure 1). The transcriptional effect of glycine but not alanine at this position might be due to the destabilizing effects of glycine substitutions on protein secondary structures.

The relative unimportance of individual acidic amino acids in VP16C is consistent with prior observations of VP16N and several other eukaryotic transcriptional activation domains (1, 6, 9, 19-21, 28, 31, 42, 45) in which the negatively charged sidechains seem relatively unimportant for transcriptional activation. For example, when four aspartate residues surrounding the critical Phe442 in VP16N were substituted by asparagine (removing the negative charge) or by glutamate (retaining the charge on a longer sidechain), the asparagine substitutions had less effect (9). Moreover, when single alanine substitutions were tested in VP16N at positions D440, D441, D443 and D445, only the D443A mutation had a significant effect (J.Kastenmeyer, P.Horn, and S.J.Triezenberg unpublished.) Thus, in both VP16C and VP16N the only acidic residue whose replacement by alanine has a significant effect is the residue immediately following the most critical phenylalanine.

In most cases, substitutions in VP16C had roughly equivalent effects when tested in yeast and in mammalian cells. This result implies that similar structural features are required for this activation domain to function in cells of these two disparate species, and might be construed to suggest that mechanisms of transcriptional activation are also conserved. An alternative hypothesis is that the specific targets of the activating domain differ in yeast and in humans, but that similar structural features of the activator are nonetheless integral to each of those distinct interactions. Careful analysis of protein:protein interactions and the transcriptional consequences of those interactions is necessary to discriminate between these two hypotheses.

Eukaryotic transcriptional activation domains bind *in vitro* to a number of putative target proteins. VP16, for example, has been shown to bind directly to TFIIA (24), TBP (18), dTAF$_{II}$40 or hTAF$_{II}$32 (15, 23), TFIIB (32), TFIIH (57), and the yeast adaptor ADA2(3). Questions remain as to which if any of these interactions are relevant to transcriptional activation *in vivo*, and whether the same hydrophobic interface is employed in each interaction. This question can be addressed in part by detailed biochemical experiments assessing the roles of activators at particular steps in the transcriptional activation process. For example, the VP16C activation domain (and others) can bind to TFIIA and can enhance the rate of formation and the stability of a complex comprising TFIIA, TFIID and promoter DNA (24). When a subset of the VP16C

mutants described in this report was tested for this property, a significant correlation was observed between the level of transcriptional activation and the ability to bind TFIIA and to stimulate formation of TFIIA/TFIID/DNA complexes (25). This result is consistent with models invoking recruitment of TFIID as an early step in the mechanism of transcriptional activation (40, 51).

Given the propensity of hydrophobic amino acid sidechains to be buried in protein tertiary structures, two simple models can be proposed for the role of such residues in transcriptional activation domains. On one hand, hydrophobic residues may be required for activation because, by interactions with each other, they facilitate formation of a structure that presents other amino acids for interaction with target proteins in the transcriptional machinery. The systematic analysis by alanine-scanning mutagenesis of the VP16C subdomain argues against this model, for we found little evidence for the critical role of any other amino acids in transcriptional activation. Alternatively, the critical hydrophobic residues may remain exposed on the surface of the activation domain to directly participate in the interaction with other proteins. These residues might then be buried in the interface created when the activation domain associates with its target.

The biophysical evidence presently available for VP16 cannot clearly distinguish between these two hypotheses, although results from studies of other activators favor the latter model. Fluorescence

spectroscopy of the VP16 activation domain revealed that key aromatic residues at positions 442 and 473 were constrained into a less flexible and more hydrophobic environment in the presence of TBP (49), but did not demonstrate that these residues make direct contact with TBP. A recent NMR study revealed that an α helix was induced in VP16C when hTAFII32 (a component of TFIID) was present (54). Of the three amino acid sidechains within this helix that showed significant chemical shifts upon interaction with TAFII32, two (F479 and L483) are bulky hydrophobic residues and the third (D472) is acidic. However, these chemical shifts do not necessarily imply that those sidechains directly participate in the interaction. Moreover, the results of this NMR analysis are at odds with the mutational analysis described in the present report, in which F473 and especially F475 are shown to play key roles in transcriptional activation whereas alanine substitutions of D472 and L483 had no apparent effect. Thus, one might have expected the NMR experiments to show pronounced chemical shifts involving the sidechains of F473 and F475. It may be that these residues are not involved in interactions with hTAFII32, but are critical for some other interaction required for transcriptional activation. Alternatively, the *in vitro* interaction of VP16C with hTAFII32 may not represent an essential step in the process of transcriptional activation. We are presently testing our panel of VP16C mutations at these positions for their effects on binding

to hTAF$_{II}$32 and other potential target proteins (Y.Nedyalkov, S.M.S., P.Horn and S.J.Triezenberg, unpublished).

Two studies offer strong evidence that the hydrophobic residues of some activating domains form surfaces that interact directly with target proteins. In a crystallographic analysis of a peptide representing the p53 transcriptional activation domain complexed with its repressor MDM2 (27), the p53 peptide folded into a helix in which transcriptionally-important hydrophobic residues formed the interacting surface. The NMR solution structure of a complex containing a peptide from the activation domain of CREB and its interacting domain from the coactivator protein CBP also showed a transition of the activation domain from random coil to amphipathic helix (41). In this case, the interaction surface involved hydrophobic residues of the activation domain and a hydrophobic groove of the coactivator, reinforced by electrostatic and hydrogen-binding interactions contributed by acidic and phosphoserine residues of the activator. We hypothesize that a similar hydrophobic surface of VP16C, encompassing the phenylalanine residues at positions 475 and 479, may interact with target proteins at key steps along the pathway to transcriptional activation, but the identification of those key steps, the relevant target proteins at those steps, and the structure of VP16 when interacting with those target proteins, await further study.

The mutational, biochemical, and biophysical studies summarized above lead to a model in which transcriptional activation by VP16 and

other eukaryotic activators requires biochemical interactions between activation domains and specific target proteins that depend most directly upon hydrophobic residues within helical segments of the activator, with relatively minor or nonspecific contributions made by acidic sidechains. The biophysical evidence that in at least some circumstances these hydrophobic residues are present in helical segments, induced by the presence of the target protein, recalls to mind the amphipathic helix model introduced early in the study of eukaryotic activators (14, 39). This model originally posited that activation domains would fold into amphipathic α-helices, the acidic face of which would comprise the interaction surface with targets such as the basic domains of TBP and TFIIB. The present evidence, while largely consistent with the formation of helices in activating domains, suggests instead that hydrophobic surfaces are key to interactions with target proteins, whereas the acidic residues may be present for bringing these otherwise buried domains to the surface, for long-range electrostatic interactions that might facilitate docking, or for minor supporting roles in the induced interaction structure. A remaining question is whether the same secondary structure and hydrophobic interaction surface will suffice for interactions of a given activator, such as VP16, with each of its various putative target proteins, and if so how this promiscuous interaction surface can nonetheless retain sufficient specificity to effectively accomplish its

transcriptional task in the complex environment of the eukaryotic nucleus.

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CHAPTER 4

MUTATIONAL ANALYSIS OF THE INTERACTION BETWEEN VP16C AND THE YEAST TRANSCRIPTIONAL ADAPTOR ADA2

INTRODUCTION

Work from multiple laboratories has indicated that maximal transcriptional activation may require proteins in addition to the transcriptional activator. Originally isolated in *Drosophila*, the TBP-associated factors (TAFs)(11) are ubiquitous among species. In yeast, at least five major adaptor complexes in addition to the TAFs are known including the mediator complex (20, 21), the SWI/SNF complex (5, 28) and its homologous complex RSC (6), the SAGA complex (14), and the ADA complex (17). SWI/SNF and mediator complexes have also been identified in mammalian systems (19, 25).

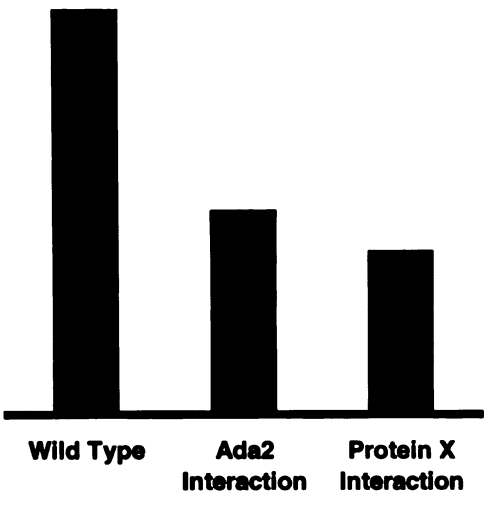
The yeast adaptor protein Ada2 is an integral part of both the ADA (7, 17, 27) and SAGA complexes (14). Originally identified in a selection for mutations that relieve toxicity of Gal4-VP16 in yeast (2), Ada2 has been shown to be required for maximal transcriptional activation by Gcn4 (9, 10), Bcl1 (3), p53 (8), retinoid X and estrogen receptors (31), and the tau1-core domain of the glucocorticoid receptor (16), and the activation domain of VP16 (30). Direct binding of Ada2 to the VP16 activation domain has been demonstrated to be a function of the C-terminal subdomain of VP16 (30). Further deletion mapping of VP16

indicated two separate Ada2 binding sites in VP16C, one in the 450-470 region and the other between residues 470-490 (1).

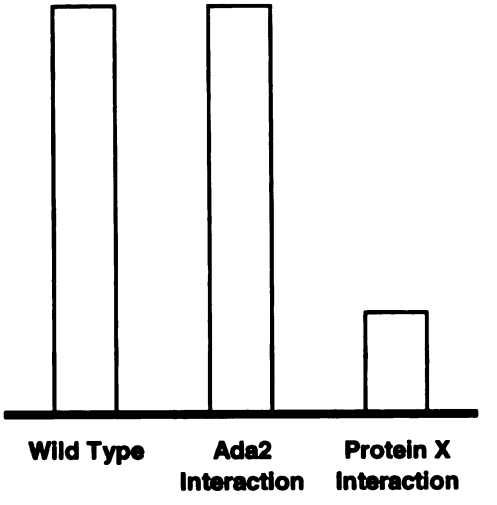
Although many adaptor complexes have been identified, the mechanism of adaptor action remains unclear. Characterization of the interaction between a transcriptional activator and a transcriptional adaptor is a step toward understanding adaptor function. An extensive set of alanine scanning and site-specific VP16C mutants (see Chapter 3) were employed to identify the amino acid residues in VP16C that are responsible for the interaction of VP16C with Ada2.

Because the *ADA2* gene is not essential in yeast (2), the activity of the VP16C mutants can be assayed in an *ada2* null background. Results of these assays are compared to the activity of the same mutants in an *ADA2* wild type strain, and the comparison is assessed as illustrated in Figure 1. In an *ADA2* wild type background (Figure 1, left hand panel), any mutation in VP16C that disrupts the interaction of VP16C and one of its *in vivo* target proteins (Ada2 or Protein X) will decrease transcriptional activation relative to wild type VP16C. In an *ada2* null background (Figure 1, right hand panel), however, only mutations that affect binding to targets in interactions that do not require Ada2 will decrease activation below wild type levels. Because activation by VP16C requires Ada2, the absence of Ada2 is functionally equivalent to the effect of a mutation that disrupts VP16C:Ada2 activity as wild type VP16C in an *ada2* null strain. Conversely, the effects of the

Figure 1. Schematic representation of the effects of various VP16C mutants on transcriptional activation in yeast. "Wild type" denotes VP16C wild type activation domain. "Ada2 Interaction" denotes a mutation in the VP16C activation domain that disrupts VP16C interaction with Ada2. "Protein X Interaction" denotes a mutation in VP16C that disrupts VP16C interaction with some other target protein. Activity of a mutant protein in a given yeast strain is expressed as a percentage of the activity of the wild type VP16C in that strain. Black bars represent activity assayed in an *ADA2* wild type yeast strain. White bars represent activity assayed in a *Δada2* yeast strain.



***ADA2* Wild Type Strain**



***Δada2* Strain**

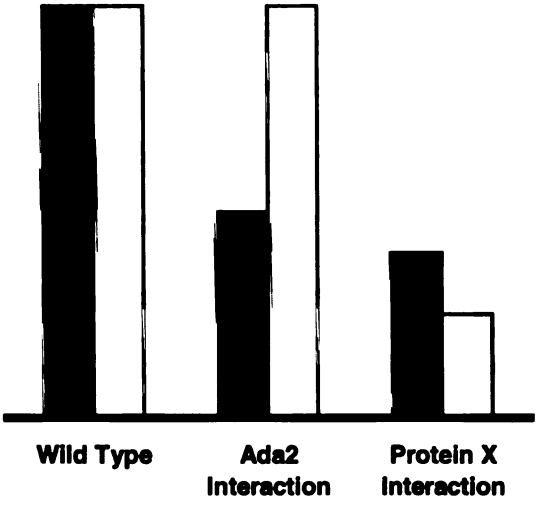


Figure 1.

absence of Ada2 and the presence of a mutation that disrupts some other interaction of VP16C (Protein X) would be additive. When results of these two assays are compared (Figure 1, bottom panel), mutations which disrupt Ada2 interaction appear to have a lower than wild type level of activity in the presence of Ada2 (black bars) and wild type activity in its absence (white bars). Conversely, mutations which disrupt interactions other than Ada2 (Protein X) do not display wild type activity in either strain, but have a higher level of activity in the presence of Ada2 than in its absence. The results of these experiments, however, failed to clearly identify specific amino acid residues in VP16C that interact with Ada2.

MATERIALS AND METHODS

Plasmids and Phage. The substrate for all mutagenesis reactions was mpPJH18, an M13mp19 phagemid containing a *Sma* I to *Bam*H I fragment encoding VP16 residues 452-490 with 119 bp of 3' untranslated sequence. Quantitative assays of transcriptional activation in yeast were performed using wild type or mutant versions of pVS1, a low copy plasmid (*CEN6 ARSH4 LEU2*) that expresses the Gal4(1-147)-VP16C fusion protein, and the yeast reporter construct pLGSD5 (15) containing the *E. coli lacZ* structural gene with the *GAL1-10* upstream activating sequence near the *CYC1* promoter.

Alanine Scanning and Site-directed Mutagenesis. Oligonucleotide-directed mutations were generated in mpPJH18 using the method described by Kunkel et al. (24). In some cases, a variety of substitutions at specific positions were generated using degenerate oligonucleotides. *Sal* I to *Bgl* II fragments containing the mutations were subcloned into pVS1 and resequenced to verify the identities of mutations.

Yeast Transformation and β-galactosidase Assay. Plasmids expressing VP16C mutants fused to the Gal4 DNA-binding domain were co-transformed with the reporter plasmid pLGSD5 into yeast strains PSY316 or PSY316*Δada2* [*MAT*a *ade2-101 his3-del.200 leu2-3,2-112 lys2 ura3-53*, *MAT*a *ada2::HisG ade2-101 his3-del.200 leu2-3,2-112 lys2 ura3-53* respectively, ref. (2)] using the procedure of Gietz *et al.* (12). To assay the activity of β-galactosidase produced from pLGSD5, 2 ml cultures containing pools of approximately 100 yeast transformant colonies were grown to early stationary phase (OD600 = 0.9-1.2) in selective media, then diluted to OD600 = 0.1, grown to mid-log phase (OD600 = 0.5) and harvested by centrifugation. β-galactosidase activity was measured (29) using extracts obtained from 5 ml cultures with results normalized to protein concentration as determined by Bradford assay (4).

RESULTS

The purpose of this set of experiments was to identify amino acid residues that are critical to the interaction of VP16C with the Ada2

protein through comparison of activities in *ADA2* wild type and Δ*ada2* yeast strains. Results of assaying the alanine scanning mutants in yeast strains PSY316 and PSY316Δ*ada2* are described in Table I. These data support the previous observation that VP16 requires Ada2 for maximal activation (30). Activity of wild type Gal4-VP16C is reduced almost tenfold in PSY316Δ*ada2* as compared to its activity in PSY316 (compare "β-gal Activity" columns). Taken as a percentage of the activity of wild type VP16C, the activity of the majority of the VP16C mutant proteins decreases 40-70% in the absence of Ada2. This is not true of mutants at positions 473, 475, 476, and 479; however, activity of those mutants is low in the *ADA2* wild type strain. Mutations at C-terminal residues 483-488 also result in a milder decrease in activation activity than the majority of the alanine changes. For these mutants, activity is only decreased 10-20% in the absence of Ada2.

When the activity of the various VP16C mutants is expressed as a percentage of the activity of wild type VP16C (Table I, Figure 2), no mutations that directly affect Ada2 interaction can be identified. The expected phenotype for Ada2 interaction mutants would be wild type activity in the Δ*ada2* strain (white bars) and lower than wild type activity in the *ADA2* wild type strain (black bars) (Figure 1). Three mutations – D469A, T480A, D481A – displayed a phenotype similar to that expected for an Ada2 interaction mutation. However, although the activity of these mutants was at a wild type level in the Δ*ada2* strain,

Table I. Relative activities in *ADA2* wild type and *Δada2* yeast strains of alanine-scanning mutants of the VP16C subdomain. Alanine substitutions were constructed at each position of VP16C except glycines and existing alanines. The activities of Gal4-VP16C fusion proteins bearing these substitutions were assayed for transcriptional activity using a Gal4-reponsive *lacZ* reporter plasmid (pLGSD5). The activites of these proteins were also assayed in yeast strain PSY316*Δada2*, a strain which lacks the adaptor protein Ada2. The position of each mutation is listed. A double substitution of alanine for both F473 and F475 is shown at the bottom of the table. Activities are expressed both in β-galactosidase units and as a percentage of the activity of wild type VP16C. Activity difference is calculated as the difference between the percentage of wild type activity in the *ADA2* wild type strain and the *Δada2* strain.

Table I.

	PSY316		PSY316Δada2		Activity
	β-gal Activity	% WT	β-gal Activity	% WT	Difference
No Activator	0 ± 0	0%	0 ± 0	0%	0
Wild Type VP16C	4700 ± 100	100%	540 ± 250	100%	0
P455A	4500 ± 100	96%	220 ± 20	41%	-55%
F457A	3600 ± 200	77%	160 ± 50	30%	-47%
T458A	4700 ± 600	100%	330 ± 50	61%	-39%
P469A	5900 ± 800	126%	300 ± 100	56%	-70%
H460A	5800 ± 600	123%	340 ± 40	63%	-60%
D461A	6300 ± 400	134%	320 ± 150	59%	-75%
S462A	5800 ± 100	123%	250 ± 40	46%	-77%
P464A	6200 ± 400	132%	310 ± 30	57%	-75%
Y465A	3600 ± 100	77%	190 ± 60	35%	-42%
L468A	3400 ± 100	72%	120 ± 40	22%	-50%
D469A	6400 ± 300	136%	790 ± 260	146%	+10%
M470A	4500 ± 100	96%	200 ± 30	37%	-59%
D472A	5900 ± 0	126%	440 ± 120	81%	-45%
F473A	2400 ± 100	51%	50 ± 20	9%	-42%
E474A	5600 ± 300	119%	460 ± 80	85%	-34%
F475A	1000 ± 100	21%	8 ± 10	1%	-20%
E476A	2000 ± 100	43%	23 ± 20	4%	-39%
Q477A	6000 ± 100	128%	380 ± 30	70%	-58%
M478A	3900 ± 800	83%	120 ± 30	22%	-61%
F479A	1800 ± 100	38%	24 ± 20	4%	-34%
T480A	4700 ± 300	100%	720 ± 180	133%	+33%
D481A	5100 ± 200	109%	920 ± 190	170%	+61%
L483A	3900 ± 100	83%	290 ± 80	54%	-29%
I485A	5300 ± 0	113%	620 ± 100	115%	+2%
D486A	4000 ± 100	85%	330 ± 80	61%	-24%
E487A	4300 ± 100	91%	430 ± 80	80%	-11%
Y488A	3900 ± 100	83%	310 ± 70	57%	-26%
F473A/F475A	0 ± 0	0%	0 ± 0	0%	0

Figure 2. Relative activities in *ADA2* wild type and *Δada2* yeast strains of alanine-scanning mutants of the VP16C subdomain. Alanine substitutions were constructed as described in Table I. The ability of these mutant VP16C proteins to activate transcription from a β-galactosidase reporter gene was assayed as described in Table I. The position of each mutation is represented by the amino acid sequence of VP16C along the horizontal axis of this figure. Bars indicate mean activity (with standard deviation) of β-galactosidase in yeast cell extracts from at least three parallel cultures, expressed relative to the activity of the wildtype Gal4-VP16C fusion protein (indicated by +). Black bars indicate the *ADA2* wild type yeast strain, white bars indicate the *Δada2* strain. β-galactosidase activities in extracts lacking the Gal4-VP16C fusion protein (indicated by -) were negligible. A double substitution of alanine for both F473 and F475 is shown at the right end of the figure.

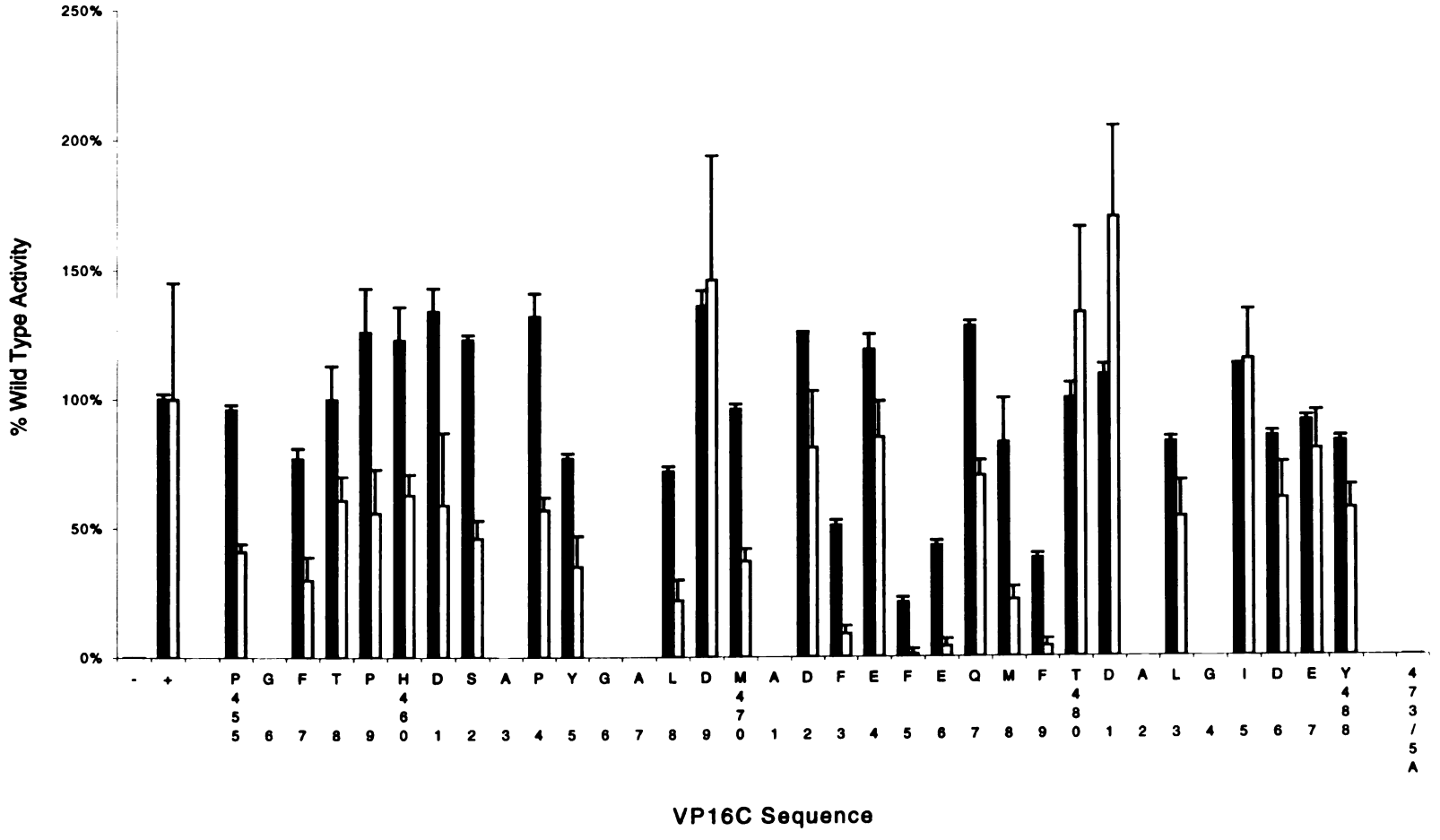

250%
200%
150%
100%
50%
0%
% Wild Type Activity
- + P455 G6 F7 T8 P9 H460 D1 S2 A3 P4 Y5 G6 A7 L8 D9 M470 A1 D2 F3 E4 F5 E6 Q7 M8 F9 T480 D1 A2 L3 G4 I5 D6 E7 Y488 473/5A
VP16C Sequence

Figure 2.

none of these changes resulted in lower than wild type activity in the presence of Ada2. It seems likely that because these mutants behave in the same manner as the wild type VP16C regardless of the presence or absence of Ada2, the residues at these positions are not involved in interaction with any *in vivo* target of VP16C.

The majority of the mutants tested demonstrated greater activity in the *ADA2* wild type strain than the Δ*ada2* strain. This phenotype is indicative of a mutation affecting transcription through an interaction that does not involve Ada2. It should be noted, however, that with the exception of F473A, F475A, E476A, and F479A, none of the mutations tested demonstrated activity in the *ADA2* wild type strain that was significantly lower than that of the wild type VP16C in the same strain. It seems that these alanine scanning mutations are not severe enough to affect transcription unless the system is otherwise affected, as by the absence of Ada2.

To further probe the most transcriptionally sensitive residues, the panels of detailed mutations at positions 473, 475, 476, 478, and 479 were also tested in the Δ*ada2* strain. The results of this analysis, shown in Figure 3, are substantially the same as those seen with the alanine scan. The majority of mutants display higher activity in the presence of Ada2 (black bars) than its absence (white bars). Moreover, the pattern seen for mutations at a given position is also the same regardless of the presence or absence of Ada2. For example, in the *ADA2* wild type strain,

Figure 3. Relative activities in *ADA2* wild type and Δ*ada2* yeast strains of Gal4-VP16C mutants bearing various amino acid substitutions at specific positions. Mutant Gal4-VP16C proteins with substitutions at F473 (panel A), F475 (panel B), E476 (panel C), M478 (panel D), or F479 (panel E), were assayed for their ability to activate expression of a β-galactosidase reporter gene as described in Table I. The activities of these proteins were also assayed in yeast strain PSY316*Δada2*, a strain which lacks the adaptor protein Ada2. The individual substitutions are indicated along the horizontal axis of each panel in descending order of activity from left to right, with the mean activity (with standard deviations) from at least three cultures for each mutant being represented by a vertical bar. Black bars indicate the *ADA2* wild type yeast strain, white bars the *Δada2* strain. Panel B also indicates the activity of one mutant with leucine substitutions at both F473 and F475.

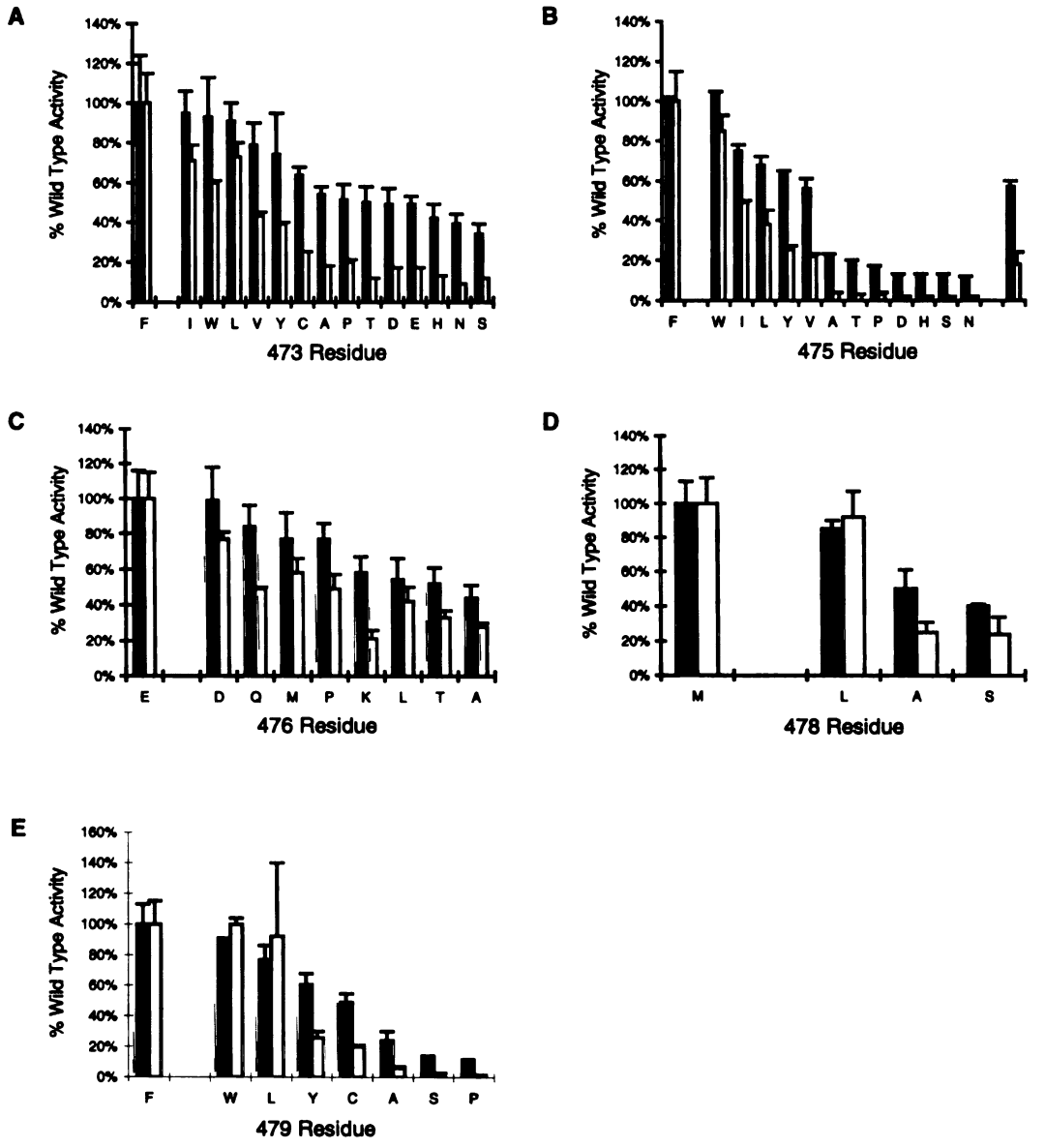

A
% Wild Type Activity
140%
120%
100%
80%
60%
40%
20%
0%
F I W L V Y C A P T D E H N S
473 Residue
B
% Wild Type Activity
F W I L Y V A T P D H S N
475 Residue
C
% Wild Type Activity
E D Q M P K L T A
476 Residue
D
% Wild Type Activity
M L A S
478 Residue
E
% Wild Type Activity
160%
F W L Y C A S P
479 Residue

Figure 3.

the native phenylalanine residue at position 475 can be replaced by bulky hydrophobic residues and retain activity, while non-hydrophobic residues disrupt activation (See Chapter 3). In the Δ*ada2* strain, the activity of all mutants is lower but the pattern of substitutions that do and do not affect transcription is the same.

In the same manner as the D469A, T480A, and D481A mutants in the alanine scanning experiment, the M478L, F479L and F479W mutants demonstrated wild type or near wild type activity levels in both yeast strains. The alanine scan established that residues 478 and 479 affect transcription via a non-Ada2 dependent pathway. These data seem to indicate that the M478L, F479L and F479W mutations do not disrupt interaction with their target protein severely enough to decrease transcription even in a system already debilitated by the absence of Ada2.

DISCUSSION

Co-immunoprecipitation experiments have shown that VP16 binds to Ada2 *in vitro* (30). Further dissection of this interaction by deletion mutagenesis and affinity chromatography demonstrated that both regions of VP16 responsible for this interaction, amino acid residues 450-470 and 470-490, lie in VP16C (1). Here we report that while the results of assaying an extensive set of VP16C point mutants *in vivo* in the absence of Ada2 support the previous observation that VP16C requires

Ada2 for maximal transcriptional activation, they provide no evidence for direct contact between amino acid residues in VP16C and Ada2 *in vivo*.

The mutational analysis does suggest evidence for direct *in vivo* interaction between VP16C and some other protein(s). Assay of VP16C mutants in yeast indicates that the side-chain of the residues at positions 473, 475, and 479 must be large and hydrophobic for maximal transcriptional activation by VP16C (Figure 3, black bars; see Chapter 3 for discussion). This suggests that these residues form a hydrophobic surface for interaction with a target protein. In an *ada2* null background, activity of these mutants is lower relative to wild type VP16C than when Ada2 is present. This additive effect of the VP16C mutation and the disruption of the *ADA2* gene suggests that VP16C is involved in at least two interactions that contribute to transcriptional activation, one that depends indirectly on Ada2 and another that depends on a target that interacts with VP16C residues 473, 475, 476 and 479. This accounts for both the decrease in transcriptional activation by VP16C in the *ada2* null strain relative to the *ADA2* wild type strain (Table I), and the transcriptionally debilitating effect of mutations at positions 473, 475, 476, and 479 that occurs regardless of the presence or absence of Ada2 (Figures 2, 3). It is also possible, however, that single amino acid substitutions are not sufficient to disrupt interaction of VP16C with Ada2 and therefore that the Ada2 interaction motif in VP16C could not be detected in these assays.

Mutations at positions 473, 475, 476, and 479 are the only point mutations in VP16C with a phenotype strong enough to be observed in a wild type yeast strain. However, the majority of the alanine scanning mutants decrease transcriptional activation in the *ada2* deleted strain. Although whatever transcriptional pathway these mutations affect does not rely directly on Ada2, it clearly is affected by the Ada2 pathway. This link may be a temporal one, such that disruption at the Ada2-dependent step in transcriptional activation decreases the efficiency of further steps. Thus the majority of VP16C mutations are mild enough that they do not affect an intact transcriptional system, but disruption of VP16C function by removal of Ada2 at an early step in transcription reduces the ability of the system to compensate for these mutations at later steps for which VP16C is also required. The observation that not all of the changes in VP16C result in an equally large reduction in activation in the absence of Ada2 may mean that certain of these residues are involved in a less important pathway than the Ada2-dependent one, or that some amino acid residues are less important for a given interaction than others.

In vitro binding experiments have identified many potential binding partners for VP16C in addition to Ada2, including TFIIA (23), TBP (18), dTAF$_{II}$40 or hTAF$_{II}$32 (13, 22), TFIIB (26), and TFIIH (32). The experiments described in this chapter indicate that not all interactions for VP16C that can be demonstrated *in vitro* occur *in vivo*. These results

confirm the value of comprehensive mutational analysis as an *in vivo* technique to verify or negate hypotheses based on *in vitro* results.

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CHAPTER 5

FLUORESCENCE SPECTROSCOPY OF THE VP16 ACTIVATION DOMAIN

INTRODUCTION

Secondary structure data for transcriptional activators has been difficult to obtain. Although in high concentrations of organic solvent or at low pH some activation domains show a preference for a particular secondary structure, activators in isolation generally appear as poorly ordered domains (11, 45, 51, 52, 62). Recently, however, a number of activation domains have been demonstrated to adopt a more ordered structure in the presence of a putative target for the activator. The activation domain of the herpes simplex virus protein VP16 has been shown by fluorescence spectroscopy to adopt a more constrained structure in the presence of TBP (53). ^{1}H-^{15}N correlated NMR techniques have shown that in the presence of $hTAF_{II}32$, a portion of the VP16 activation domain adopts an α-helical structure (61), as does an activating domain of CREB with a portion of CBP (47). Likewise, circular dichroism demonstrates induced secondary α-helical structure for the transcription factor c-myc in the presence of TBP (43). X-ray crystallography has shown that the p53 transcriptional activation

domain adopts an amphipathic α-helical structure in the presence of its repressor MDM2 (34).

Mutational analyses of a large number of transcriptional activators suggest that the most critical residues for transcriptional activation are also bulky hydrophobic and aromatic amino acids (1, 4, 9, 13, 23, 24, 38, 40, 48, 50). These residues may be critical because of their role in providing a surface for interaction with a target protein through which secondary structure can be induced. The x-ray crystal structure of the p53-MDM2 complex, for example, indicates that the surface of p53 that interfaces with MDM2 consists of a Trp, a Phe, and a Leu residue (34) previously shown to be critical for transcriptional activation (40). In this report, we investigate a potential correlation between mutations that disrupt activation ability in VP16 and the formation of TBP-induced structure.

Previous work has shown that the activation domain of VP16 (residues 413-490) can be divided into two subdomains. VP16N consists of amino acids 412 to 450 and VP16C comprises amino acids 450 to 490 (15, 45, 60, 63). Each of these subdomains is independently capable of activation when provided with a DNA binding mechanism. Mutational analyses have demonstrated that the most critical residue for activation by VP16N is a Phe at position 442, with some assistance from flanking leucines at positions 439 and 444, and an aspartate at position 443 (45,

9; J.Kastenmeyer, P.J.Horn, and S.J.Triezenberg, unpublished). In VP16C, the most critical residues are Phe 475 and Phe 479, with some contribution by Phe 473 and Glu 476 (56). Any of these Phe residues can be replaced by Trp without eliminating activity, but replacement with Ala severely decreases transcriptional ability.

In this chapter I describe a fluorescence analysis of chimeric proteins consisting of the DNA binding domain of the yeast protein Gal4 and the activation domain of VP16. Trp codons were substituted for Phe in the VP16 coding region to provide a unique intrinsic fluorescence probe. During protein synthesis in *E. coli*, the 5-hydroxytryptophan analogue was incorporated in place of normal Trp into the Gal4-VP16 protein. The excitation spectrum of 5-OH-Trp is shifted to longer wavelengths than that of native Trp, thus allowing selective excitation of 5-OH-Trp in the presence of normal Trp residues. Previous analysis by fluorescence spectroscopy has shown that Phe 442 and Phe 473 are solvent-exposed in highly mobile segments which become constrained in the presence of TBP (52, 53). Here we observe that while this is also true for Phe 475, the binding of the VP16 activation domain to TBP is unaffected by mutations that disrupt transcriptional activation.

In addition, a 5-hydroxytryptophan probe was used to study the effect of TATA-box containing DNA on the interaction between TBP and VP16. X-ray crystal structures for TBP bound to DNA have been solved

(25, 28), and x-ray crystallography, photo-crosslinking, and mutational analysis have provided structural information about TBP complexed with DNA and other basal transcription factors (12, 35, 44, 49, 58). Mutational analysis of TBP has also allowed identification of TBP residues that affect the activity of transcriptional activators (2, 26, 27, 37, 39, 59). However, to date no structural information exists on the association of transcriptional activators with TBP in the presence of TATA-box DNA.

Pugh and colleagues have evidence that suggests that both TBP and TFIID exist as dimers in solution, raising the possibility that a function of transcriptional activators with respect to TBP is to dissociate the TBP dimer thus allowing TBP to interact with TATA-box DNA (7, 8, 57). Mutational analysis of several transcriptional activators has demonstrated a correlation between the ability to bind TBP and the ability to activate transcription (2, 37). Moreover, a mutation in TBP that disrupts transcriptional activation by VP16 can be found on the DNA binding surface of TBP (26) and VP16 has been suggested to induce a conformational change in TBP that affects binding of TBP to TATA (39). Thus it is possible that one of the roles of VP16 in activating transcription is to dissociate the TBP dimer, perhaps by binding to TBP, thereby facilitating TBP binding to DNA. To explore the potential relationship between TBP:DNA and TBP:VP16 interactions, we used

fluorescence anisotropy to determine whether a TBP-VP16 complex could be dissociated by addition of TATA-box DNA. The results indicate that TATA-box DNA may dissociate the VP16-TBP complex, suggesting an equilibrium between TBP binding to VP16 and the TATA-box.

MATERIALS AND METHODS

Mutagenesis and cloning. The Gal4-VP16 proteins used in these experiments are described in Table I. Plasmids pFS12182(WV36).F442W and pFS12182(WV36).F473W were previously constructed by Fan Shen (52). Plasmids pVS1.F475A and pVS1.F475W were previously constructed (56). All derivatives of yeast ARS/CEN plasmid pTH2-1 contain the full length VP16 activation domain coding region with the specified mutations fused to the wild type Gal4-DNA binding domain coding region. All derivatives of yeast ARS/CEN plasmid pVS1 contain the VP16C activation domain coding region with the specified mutations fused to the wild type Gal4 DNA binding domain coding region. All plasmids designated pSMS156 are *E. coli* expression vectors containing the Gal4 DNA binding domain coding region (coding for amino acid residues 1-147) with the WV36 mutation fused to the full length VP16 activation domain coding region (coding for amino acid residues 411-490) with the listed mutations.

Table I. Gal4-VP16 constructs used in this study. All proteins used contain the Gal4 DNA binding domain (amino acids 1-147) with a tryptophan to valine substitution at position 36. All proteins also contain a two amino acid (Pro-Gly) linker between the Gal4 and VP16 domains. All proteins contain the activation domain of VP16 (amino acids 411-490) with 5-hydroxytryptophan and alanine substitutions as indicated.

Construct	5-hydroxytryptophan substitution	Alanine substitution
442W	Phe 442	none
442W/444A	Phe 442	Leu 444
442W/475A	Phe 442	Phe 475
473W	Phe 473	none
473W/475A	Phe 473	Phe 475
475W	Phe 475	none
475W/479A	Phe 475	Phe 479
442A/475W	Phe 475	Phe 442

F442W/L444A: Oligonucleotide directed mutagenesis of a VP16N M13 template (derived from mpDC3) that carries an L444A mutation was used to create the F442W/L444A double mutant in the VP16N subdomain coding region (33). The VP16N subdomain coding region from mpDC3.F442W/L444A was then cloned into pTH2-1 using *Ava*I and *Sal*I to generate the insert, and *Psp*AI and *Sal*I to generate the vector (*Ava*I and *Psp*AI produce compatible ends). This yielded pTH2-1.F442W/L444A. The VP16 activation domain coding region from pTH2-1.F442W/L444A was cloned into the *E. coli* expression plasmid pFS12182(WV36).F473W, which carries the valine for tryptophan mutation in the Gal4 DNA binding domain coding region, using *Xho*I and *Hin*dIII. The resulting plasmid was designated pSMS156.F442W/L444A.

F442W/F475A: The VP16C subdomain coding region from pVS1.F475A was cloned into the *E. coli* expression plasmid pFS12182(WV36).F442W, which carries the valine for tryptophan mutation in the Gal4 DNA binding domain coding region, using *Ava*I and *Hin*dIII to generate the insert, and *Psp*AI and *Hin*dIII to generate the vector. The resulting plasmid was designated pSMS156.F442W/F475A.

F473W/F475A: Oligonucleotide directed mutagenesis of the VP16C M13 template mpPJH18.F475A was used to create the F473W/F475A double mutant in the VP16C subdomain coding region (33). The VP16C subdomain coding region from mpPJH18.F473W/F475A was then cloned

into pTH2-1 with *Sal*I and *Bgl*II to yield pVS1.F473W/F475A. The VP16C activation domain coding region from pVS1.F473W/F475A was cloned into pFS12182(WV36).F473W using *Ava*I and *Hin*dIII to generate the insert and *Psp*AI and *Hin*dIII to generate the vector. The resulting plasmid was designated pSMS156.F473W/F475A

F475W: The VP16C activation domain coding region from pVS1.F475W was cloned into pFS12182(WV36).F473W using *Ava*I and *Hin*dIII to generate the insert and *Psp*AI and *Hin*dIII to generate the vector. The resulting plasmid was designated pSMS156.F475W.

F475W/F479A: Oligonucleotide directed mutagenesis of the VP16C M13 template mpPJH18.F475W was used to create the F475W/F479A double mutant in the VP16C subdomain coding region (33). The VP16C subdomain coding region from mpPJH18.F475W/F479A was then cloned into pTH2-1 with *Sal*I and *Bgl*II to yield pVS1.F475W/F479A. The VP16C activation domain coding region from pVS1.F475W/F479A was cloned into pFS12182(WV36).F473W using *Ava*I and *Hin*dIII to generate the insert and *Psp*AI and *Hin*dIII to generate the vector. The resulting plasmid was designated pSMS156.F475W/F479A.

F442A/F475W: The VP16N subdomain coding region carrying an F442A mutation was cloned from pACF442A into pFS12182(WV36).F473W using *Ava*I. Clones were checked for orientation, and the plasmid was designated pSMS156.F442A/F473W.

The VP16C subdomain coding region from pVS1.F475W was cloned into pSMS156.F442A/F473W using *Psp*AI and *Hind* III. The resulting plasmid was designated pSMS156.F442A/F475W.

TATA-box oligonucleotide design and creation. Two twenty base oligonucleotides, ST274 (5'-CTGTATGTATATAAAAC-3') and ST275 (5'-GTTTTATATACATACAG-3'), corresponding to the yeast CYC1 promoter TATA box (12) were synthesized. Oligonucleotides were lyophilized and resuspended to a concentration of 1 μM in TE. Equal volumes of ST274 and ST275 were annealed by heating to 90°C and allowing the mixture to cool slowly to room temperature to produce 1μM TATA-box DNA.

Expression and purification of proteins. Yeast TBP was purified from *E. coli* BL21(DE3) cells carrying the plasmids pLysS and pKA9 (a gift from Dr.Fred Winston) using the procedure of Petri, *et al.* (46) as modified by Shen, *et al.* (53). Yeast TBP used in these experiments was a gift from Fan Shen. To incorporate 5-hydroxytryptophan into Gal4-VP16 proteins, *E.coli* strain CY15077 containing Gal4-VP16 encoding plasmids were grown and harvested as previously described (52). Proteins were purified using a modified version of the procedure of Shen, *et al.* (53). Cell pellets were resuspended in 40 ml per liter of cell culture of Buffer A_{200} (20 mM HEPES, pH 7.5, 10 μM zinc acetate, 200 mM NaCl, 20 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml benzamadine, 2 μg/ml leupeptin, 2μl/ml pepstatin). The cells were lysed

by sonication and the cell debris removed by centrifugation. Polyethylenimine was added to the cleared lysate to 0.06% (v/v) and the precipitated proteins were removed by centrifugation. Ammonium sulfate was added to the supernatant to 25% w/v. The precipitated proteins were resuspended in 1.67 ml per 1 L of cell culture of 10 mM HEPES, pH 8.5 and the solution was cleared by centrifugation at 14,000 rpm for 20 min. The resulting supernatant was separated by preparative HPLC (Waters 600E controller; TOSOHAAS (TOYO SODA) TSK DEAE-5PW column, 21.5mmx150mm). The column was washed with 10 mM HEPES, pH 8.5 and eluted with a linear salt gradient (187-375 mM KCl in 10 mM HEPES, pH 8.5). Fractions containing the purified proteins, as judged by SDS-PAGE, were pooled and stored as aliquots at –70°C.

In vitro transcription assays. Yeast TBP activity was tested in *in vitro* transcriptional assays using HeLa nuclear extracts as described (6, 53).

Gel mobility shift assays. A 17 basepair double-stranded oligonucleotide bearing a consensus binding site for yeast protein Gal4 was created by annealing 100 ng of oligonucleotide ST23 (5'-CGGAAGACTCTCGTCCG-3') and ^{32}P-labeled oligonucleotide ST24 (5'-GCCTTCTGACAGCAGGC-3'). A 30 µl reaction mixture containing approximately 10 fmol of ^{32}P-labelled DNA, 5 µg (170 pmol) Gal4-VP16 wild type or mutant protein, 12.5 mM Hepes, pH 7.5, 60 mM KCl, 12.5% glycerol, 5 mM $MgCl_2$, and 10mM β-mercaptoethanol was incubated at room temperature for 1 hour.

Reactions were separated on 15 x 18 cm 4% polyacrylamide gels. Gels were electrophoresed in 1xTTE at 100V for approximately 1.5 hours. Gels were dried and visualized via autoradiography.

Fluorescence measurements. All proteins were dialyzed against phosphate buffered saline (pH 7.4, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) with 8% (v/v) glycerol. Concentrations of Gal4-VP16 samples were determined using the extinction coefficient derived from amino acid composition (14, 21), $\varepsilon_{280} = 9460\ cm^{-1}M^{-1}$. Absorbance measurements were obtained using a HP8452A diode array spectrophotometer. Concentrations of all proteins used in this study were 2 μM. The optical density of these solutions was less than 0.1 at the excitation wavelength to avoid inner filter effects.

Steady state anisotropy measurements were obtained on a Jobin Yvon-Spex Instruments SA, Inc. Fluormax2 spectrophotometer using an L-format detection configuration. The excitation wavelength was 309 nm and the bandwidth was 4nm. The emission wavelength was 360 nm and the bandwidth was 8 nm. Every data point was measured at least 6 times. Data were fit to the equations describing the formation of the 1:1 binary complex between Gal4-VP16 and TBP and K_D values were determined using least-squares regression (Scientist 2.0 for Windows, MicroMath Scientific Software, Salt Lake City, UT).

Time-resolved anisotropy decay of Gal4-VP16 and Gal4-VP16:TBP complexes was measured on a single photon counting fluorometer (16). A synchronously pumped, mode-locked, cavity-dumped dye laser (Spectra-Physics 3520) was used as the light source, with an excitation wavelength of 308 nm. Anisotropy decay curves were obtained by alternately recording emission oriented parallel and perpendicular to the plane of excitation at an emission wavelength of 360 nm. A glass slide was added to further reject scattered light; however, both raman and raleigh scatter were higher than previously observed (52, 53) so that r_o(app), might be somewhat contaminated by scatter. Time per channel was 85 ps, and 512 channels were recorded. Data were analyzed by the "sum and difference" method (10) using the Decayfit software developed in the Knutson laboratory. The anisotropy decay curve, $r(t)$, was obtained from the difference curve an total intensity curve by the following,

$$r(t) = (I_{vv}-I_{vh})/(I_{vv} + 2I_{vh}) \qquad \text{(Eq.1)}$$

where I_{vv} and I_{vh} are emission intensities measured parallel and perpendicular to the excitation plane, respectively. The system depolarizer makes the G factor equal to unity. $r(t)$ was modeled by a sum of exponentials,

$$r(t) = \Sigma B_j exp(-t/\phi_j) \qquad \text{(Eq.2)}$$

where ϕ_j is the rotational correlation time of the jth component, and β_j is its preexponential term. If one assumes segmental motion can be

reconciled with the "wobbling in cone" model (29, 42), the cone semiangle, θ, is given by the following,

$$\beta_2 = r_o[1/2(\cos\theta)(1 + \cos\theta)^2 \quad \text{(Eq.3)}$$

where β_2 is the preexponential term for the global rotation of the macromolecule and r_o is the limiting ("time zero") anisotropy.

Gal4-VP16:TBP:TATA-box DNA experiments were carried out using solutions containing 2 μM Gal4-VP16.F442W and 4 μM TBP in PBS/8% glycerol. TATA-box DNA in TE was titrated from 0-6.9 μM. Time-resolved anisotropy decay of Gal4-VP16:TBP:TATA-box DNA complexes was measured on a 16 channel time correlated single photon counting fluorometer coupled to a multianode microchannel plate photomultiplier tube (HR R3841-07). A synchronously pumped, mode-locked, cavity-dumped dye laser (Spectra-Physics 3520) was used as the light source, with an excitation wavelength of 308 nm. Emitted light was split by a Rochone prism according to polarization and introduced into an imaging spectrometer (Spex 270M). This system was designed and built by J.Xiao in the laboratory of J.Knutson at the National Institutes of Health.

RESULTS

Production of Gal4-VP16 fusion proteins with unique 5-OH-tryptophan substitutions and phenylalanine to alanine mutations. Gal4-VP16 fusion proteins containing 5-OH-tryptophan were created to provide a unique

intrinsic fluorophore with which to study the structure induced in VP16 activation domain by interaction with TBP. The chimeric transactivator proteins used in this study contained the DNA-binding domain of the yeast protein Gal4 (residues 1-147) fused to wild type or mutant activation domains of VP16 (residues 411-490) by a two amino acid (Pro-Gly) linker. The original Gal4 protein had a tryptophan residue at position 36, which was replaced by valine with no effect on DNA binding (52). The native activation domain of VP16 has no tryptophan residues. To insert unique fluorescence probes in the VP16 activation domain, the codons for phenylalanine residues at positions 442, 473, or 475 were replaced by tryptophan codons in an *E. coli* expression plasmid. A tryptophan auxotrophic *E. coli* strain containing the various expression plasmids was grown in the presence of 5-hydroxytryptophan to incorporate the 5-OH-Trp analogue at these positions.

In addition to the 5-OH-tryptophan substitutions, versions of each of these proteins bearing transcription debilitating substitutions were also constructed. Alanine codons were inserted in place of Phe442, Leu444, Phe475, and Phe479 in combination with the Trp substitutions to create the panel of proteins described in Table I. Mutational analysis of Gal4-VP16N or Gal4-VP16C indicates that alanines placed at positions 442, 444, 475, or 479 disrupt transcriptional activation *in vivo* (48, 56). By combining transcription debilitating mutations with 5-OH-Trp probes,

we hoped to observe the effects of changes at these positions on the TBP-induced structure of VP16 activation domain. The F442W/L444A, F473W/F475A, and F475W/F479A combinations were designed to provide information about potential structural changes as observed from the same subdomain as the transcriptional mutation. The F442W/F475A and F442A/F475W pairs were designed to test whether mutations in one subdomain which affect transcriptional activation would also affect TBP induced structure in the other subdomain.

To ensure that the VP16 mutations were not affecting DNA binding, gel mobility shift assays were performed. All of the Gal4-VP16 proteins were capable of binding to DNA at or near the level of the wild type Gal4-VP16 (Figure 1).

Steady state excitation and emission spectra were analyzed to ensure that 5-hydroxytryptophan had been incorporated into the purified Gal4-VP16 proteins. Excitation spectra were characteristic of 5-hydroxytryptophan (20) and did not differ significantly between transcriptional mutants and their respective wild types (Figure 2A-C). Emission spectra for all eight proteins tested demonstrated a peak at 335 nm, close to that of the free amino acid analogue (20) (Figure 3A-C). This supports the previous observation that the fluorophores placed at these positions are largely solvent exposed (52, 53). Moreover, the emission spectra (Figure 3A-C, compare dotted or dashed lines to solid) indicate

Figure 1. Gel mobility shift assay with Gal4-VP16 proteins. A double stranded oligonucleotide ^{32}P-labeled containing a Gal4 binding site was incubated with wild type Gal4-VP16 or mutant Gal4-VP16 proteins bearing the W36V change in Gal4 and the 5-OH-tryptophan and alanine substitutions in the activation domain. Proteins are identified by mutation, with "W" indicating 5-OH-tryptophan.

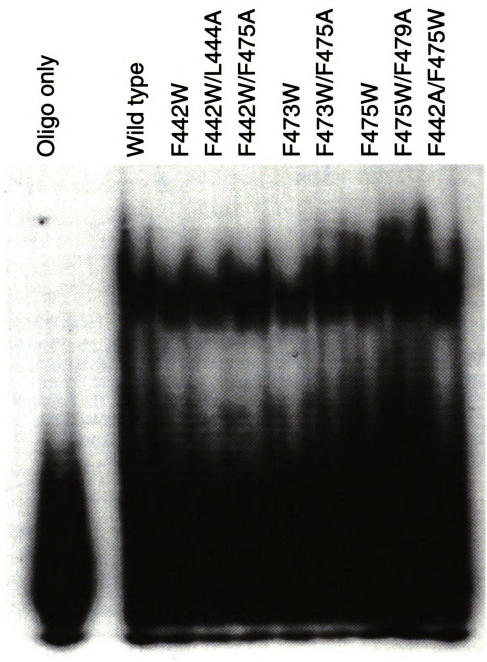
Oligo only
Wild type
F442W
F442W/L444A
F442W/F475A
F473W
F473W/F475A
F475W
F475W/F479A
F442A/F475W

Figure 1.

Figure 2. Excitation spectra of Gal4-VP16 proteins labeled with 5-hydroxytryptophan. Emission wavelength 360 nm. A. Proteins with probe at position 442. 442W alone: solid line. 442W/444A: dotted line. 442W/475A: dashed line. B. Proteins with probe at position 473. 473W alone: solid line. 473W/475A: dotted line. C. Proteins with probe at position 475. 475W alone: solid line. 475W/479A: dotted line. 442A/475W: dashed line. "W" denotes 5-OH-tryptophan.

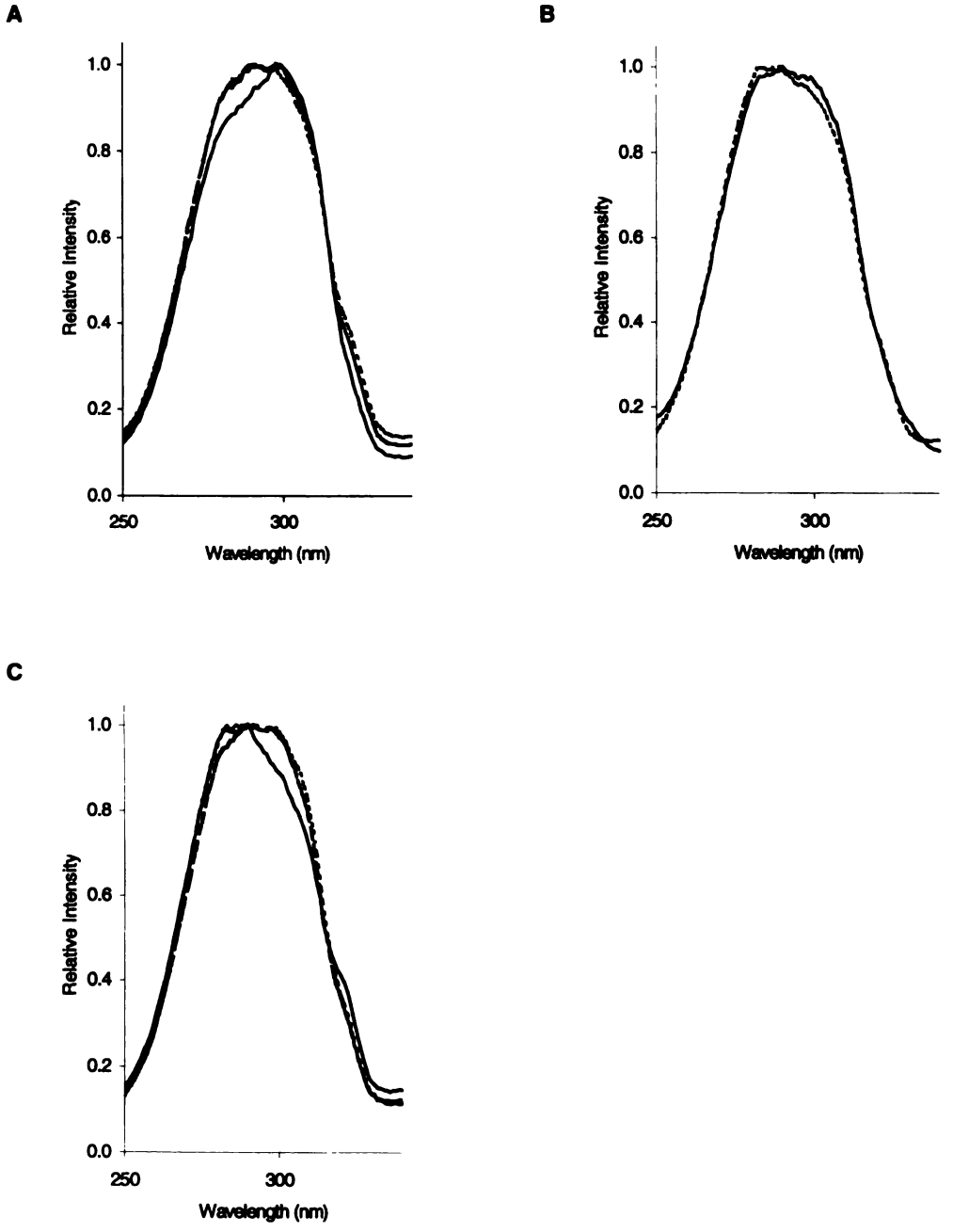
A
B
C
Relative Intensity
1.0
0.8
0.6
0.4
0.2
0.0
250
300
Wavelength (nm)

Figure 2.

Figure 3. Emission spectra of Gal4-VP16 proteins labeled with 5-hydroxytryptophan. Excitation wavelength was 310 nm. A. Proteins with fluorescent probe at position 442. 442W alone: solid line. 442W/444A: dotted line. 442W/475A: dashed line. B. Proteins with probe at position 473. 473W alone: solid line. 473W/475A: dotted line. C. Proteins with probe at position 475. 475W alone: solid line. 475W/479A: dotted line. 442A/475W: dashed line. "W" denotes 5-OH-tryptophan.

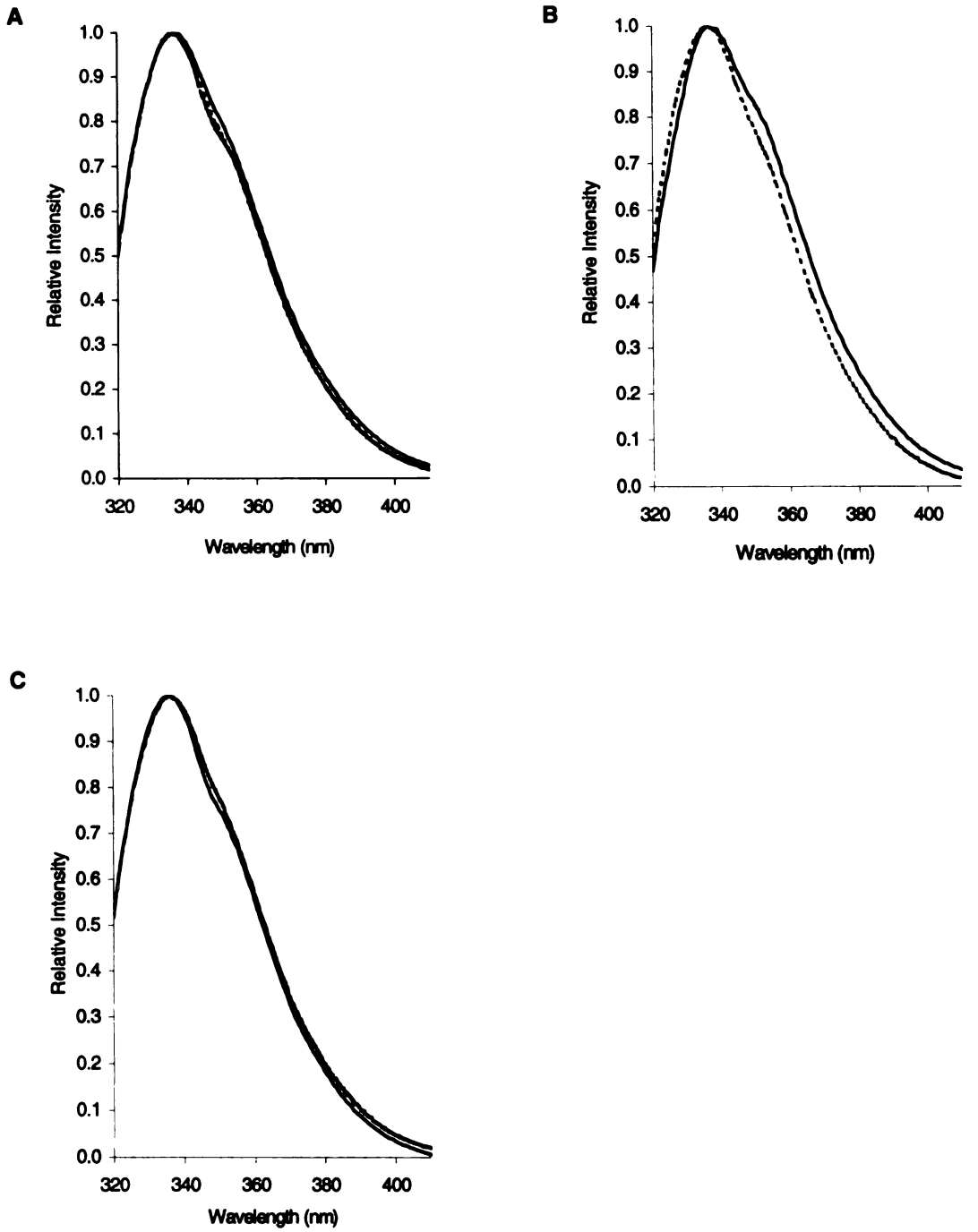
A
B
C
Relative Intensity
Wavelength (nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
320
340
360
380
400

Figure 3.

that the various activation mutations have no effect on the measured fluorescence properties of the 5-OH-tryptophan probe.

Steady state fluorescence anisotropy and dissociation constants for the interaction of the VP16 activation domain mutants and TBP. Previous work had shown an increase in steady-state anisotropy of Gal4-VP16 protein in the presence of TBP, indicative of a complex between the two (53). To determine whether VP16 amino acids that are important for transcriptional activation are also important for interaction with TBP, steady state anisotropy experiments were performed using 5-hydroxytryptophan labeled Gal4-VP16 proteins with or without an activation mutation. Anisotropy values were measured upon incremental addition of TBP and values were plotted as a function of TBP concentration.

The results (Figure 4) indicate that activation mutants at positions 444 and 475 have no discernible effect on the interaction of Gal4-VP16 with TBP. The measured anisotropy values and the calculated binding curves for the proteins bearing the L444A or F475A substitutions in conjunction with the 5-OH-Trp substitution at position 442 do not differ significantly from those of the protein bearing the position 442 5-OH-Trp alone (Figure 4A). Within the limits of experimental error, anisotropy values measured for all three proteins are indistinguishable. Binding curves fit by least-squares regression were nearly identical for all three

Figure 4. Steady-state anisotropy analysis of Gal4-VP16 proteins in the presence of TBP. A. Proteins with probe at position 442. 442W alone: diamonds, solid line. 442W/444A: squares, dotted line. 442W/475A: circles, dashed line. B. Proteins with probe at position 473. 473W alone: diamonds, solid line. 473W/475A: squares, dotted line. C. Proteins with probe at position 475. 475W alone: diamonds, solid line. 475W/479A: squares, dotted line. 442A/475W: circles, dashed line. Data represent the average of three separate experiments. "W" denotes 5-OH-tryptophan. Lines represent best fit curves derived from the data points by least squares regression for a presumed 1:1 binary complex between Gal4-VP16 and TBP.

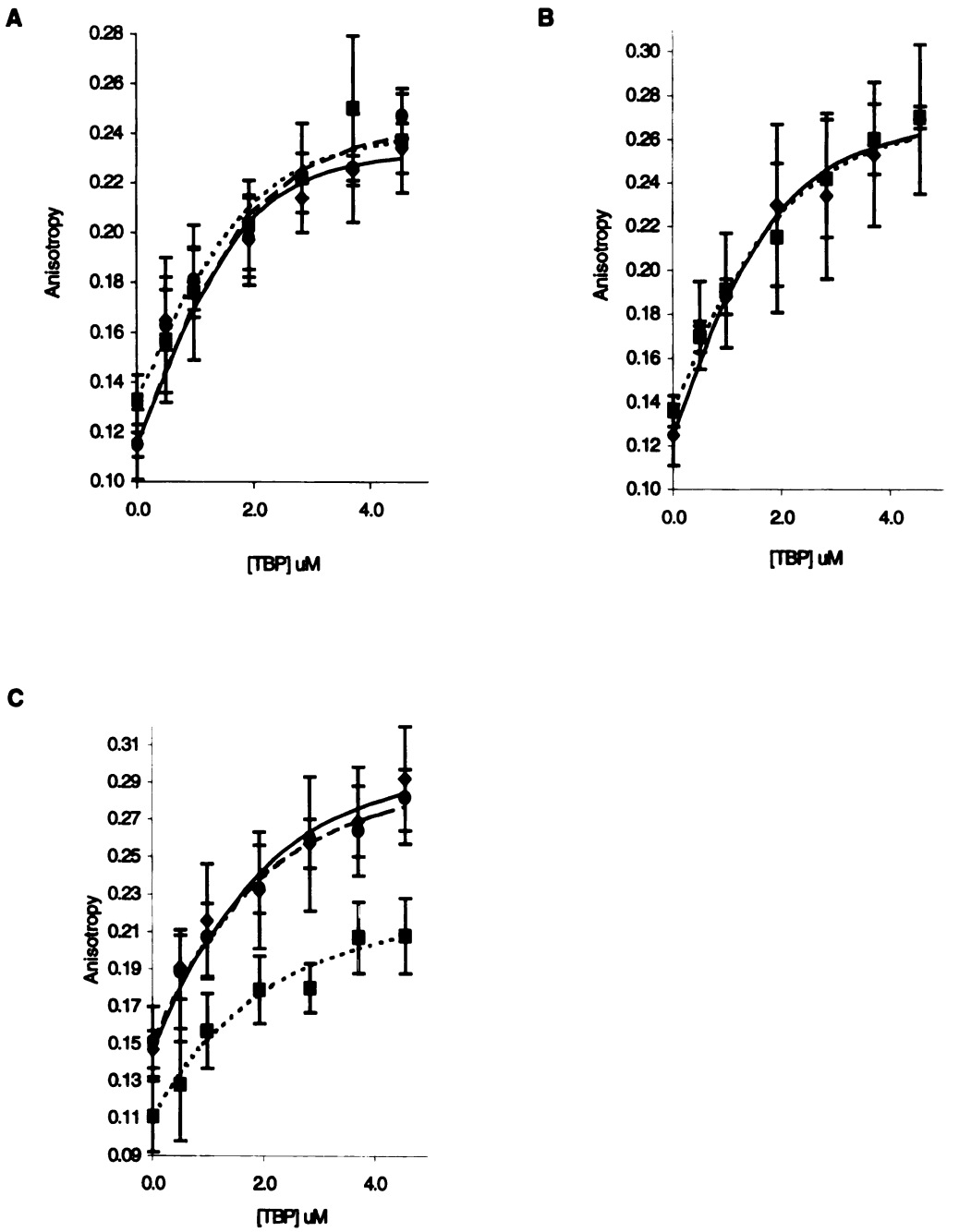
A
Anisotropy
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.0
2.0
4.0
[TBP] uM
B
Anisotropy
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.0
2.0
4.0
[TBP] uM
C
Anisotropy
0.31
0.29
0.27
0.25
0.23
0.21
0.19
0.17
0.15
0.13
0.11
0.09
0.0
2.0
4.0
[TBP] uM

Figure 4.

proteins carrying the fluorophore at residue 442. Dissociation constants calculated from the binding curves indicated that the activation mutations do not significantly alter the interaction between TBP and Gal4-VP16 (Table II). The value of $4 \pm 1 \times 10^{-7}$ M calculated for the proteins bearing the 444A and 475A mutations does not differ significantly from the $3 \pm 1 \times 10^{-7}$ M constant calculated for the 442 fluorophore alone. Moreover, both of these dissociation constants are consistent with the $3.3 \pm 1.7 \times 10^{-7}$ M value previously reported (53). The 444A and 475A mutations do not alter binding of TBP and Gal4-VP16 as detected by a fluorescent probe at the 442 position.

The F475A mutation in conjunction with a probe at position 473 also has no effect on TBP binding. The anisotropy values measured for the 475A mutation in the context of a 473 fluorophore and the corresponding binding curves are almost identical to those measured for the fluorophore alone (Figure 4B). K_D values calculated for each of these proteins are similar (Table II). The 473 probe alone has a dissociation constant of $5 \pm 1 \times 10^{-7}$ M, while the 475A mutant has a value of $7 \pm 1 \times 10^{-7}$ M. The difference in these values is not statistically significant. It should be noted, however, that the measured K_D value of $5 \pm 1 \times 10^{-7}$ M for the 473 probe alone is significantly higher than the $2.8 \pm 0.6 \times 10^{-8}$ M previously reported (53).

Table II. Calculated dissociation constants from steady state anisotropy experiments. Best fit lines for the 1:1 binary complex between Gal4-VP16 and TBP were derived by least squares regression. K_D values were calculated from the best fit line.

Construct	K_D
442W	$3 \pm 1 \times 10^{-7}$ M
442W/444A	$4 \pm 1 \times 10^{-7}$ M
442W/475A	$4 \pm 1 \times 10^{-7}$ M
473W	$5 \pm 1 \times 10^{-7}$ M
473W/475A	$7 \pm 1 \times 10^{-7}$ M
475W	$1 \pm 0.2 \times 10^{-6}$ M
475W/479A	$1 \pm 0.2 \times 10^{-6}$ M
442A/475W	$1 \pm 0.1 \times 10^{-6}$ M

The values measured for the 442A mutation in conjunction with a 5-OH-Trp at position 475 do not vary from those of the 475 fluorophore alone (Figure 4C). Moreover, the calculated K_D value is $1 \pm 0.2 \times 10^{-6}$ M for both the 475 probe and the 475 probe with the 442A mutation (Table II). This mutation does not discernibly affect the binding of TBP to VP16.

The only alanine change that seemed to have some effect on steady state anisotropy was the F479A mutation (Figure 4C). The measured values were significantly lower for the protein bearing both the F479A mutation and the 475 probe than for the 475 probe by itself. However, the K_D for the 479A mutant protein was $1 \pm 0.1 \times 10^{-6}$ M, the same as that of the 475 probe alone (Table II). This indicates that binding of TBP in fact is not affected by the 479A mutation. The lower anisotropy values for this protein are most likely to result from differences in the local environment of the fluorophore (see Discussion).

As described, the K_D values for all activation mutants tested were the same as those of their respective fluorophores alone (Table II). Interestingly, however, fluorophores at different positions had different K_D values. The proteins with fluorophores at position 475 seemed to bind slightly less strongly than those with position 442 or 473 fluorescent probes.

Time resolved anisotropy and segmental motion of the VP16 activation domain when complexed with TBP. Time resolved anisotropy studies were

also undertaken to confirm and expand the results of the steady-state experiments. Previous work indicated a decrease in segmental motion for probes at both 442 and 473 in the presence of TBP (53). Anisotropy decay curves were obtained for all proteins in the presence and absence of a twofold molar excess of TBP. Decay parameters for proteins bearing transcriptionally debilitating mutations were compared to those for proteins with the probe alone to determine if these mutations would alter the nature of the binding of TBP to VP16.

The anisotropy decay curves of the Gal4-VP16 proteins in the presence and absence of TBP are shown in Figure 5. For all proteins tested, the addition of TBP slowed the anisotropy decay. In all cases, regardless of the presence of an activation mutation, the decay curves in the presence of TBP (Figure 5A-E, light dotted and solid lines) retain higher anisotropy values for longer times than those of the Gal4-VP16 proteins alone (Figure 5A-E, heavy dotted and solid lines). This indicates that, as shown by the steady state anisotropy data, the mutations that debilitate transcriptional activity do not eliminate the binding to TBP.

The anisotropy decay curves for proteins bearing activation mutations appear similar to those of their respective probes alone (Figure 5). The curves for all mutant proteins overlap those of probe alone in the absence of TBP (Figure 5A-E, light dotted and solid lines). Moreover, for these proteins in the presence of TBP, the curves also

Figure 5. Time-resolved anisotropy decay curves for Gal4-VP16 proteins in the presence and absence of TBP. 2 μM activators and 4 μM TBP were used in these experiments. A. 442W: solid line. 442W/444A: dotted line. 442W+TBP: heavy solid line. 442W/444A+TBP: heavy dotted line. B. 442W: solid line. 442W/475A: dotted line. 442W+TBP: heavy solid line. 442W/475A+TBP: heavy solid line. C. 473W: solid line. 473W/475A: dotted line. 473W+TBP: heavy solid line. 473W/475A+TBP: heavy dotted line. D. 475W: solid line. 475W/479A: dotted line. 475W+TBP: heavy solid line. 475W/479A+TBP: heavy dotted line. E. 475W: solid line. 442A/475W: dotted line. 475W+TBP: heavy solid line. 442A/475W+TBP: heavy dotted line. "W" denotes 5-OH-tryptophan.

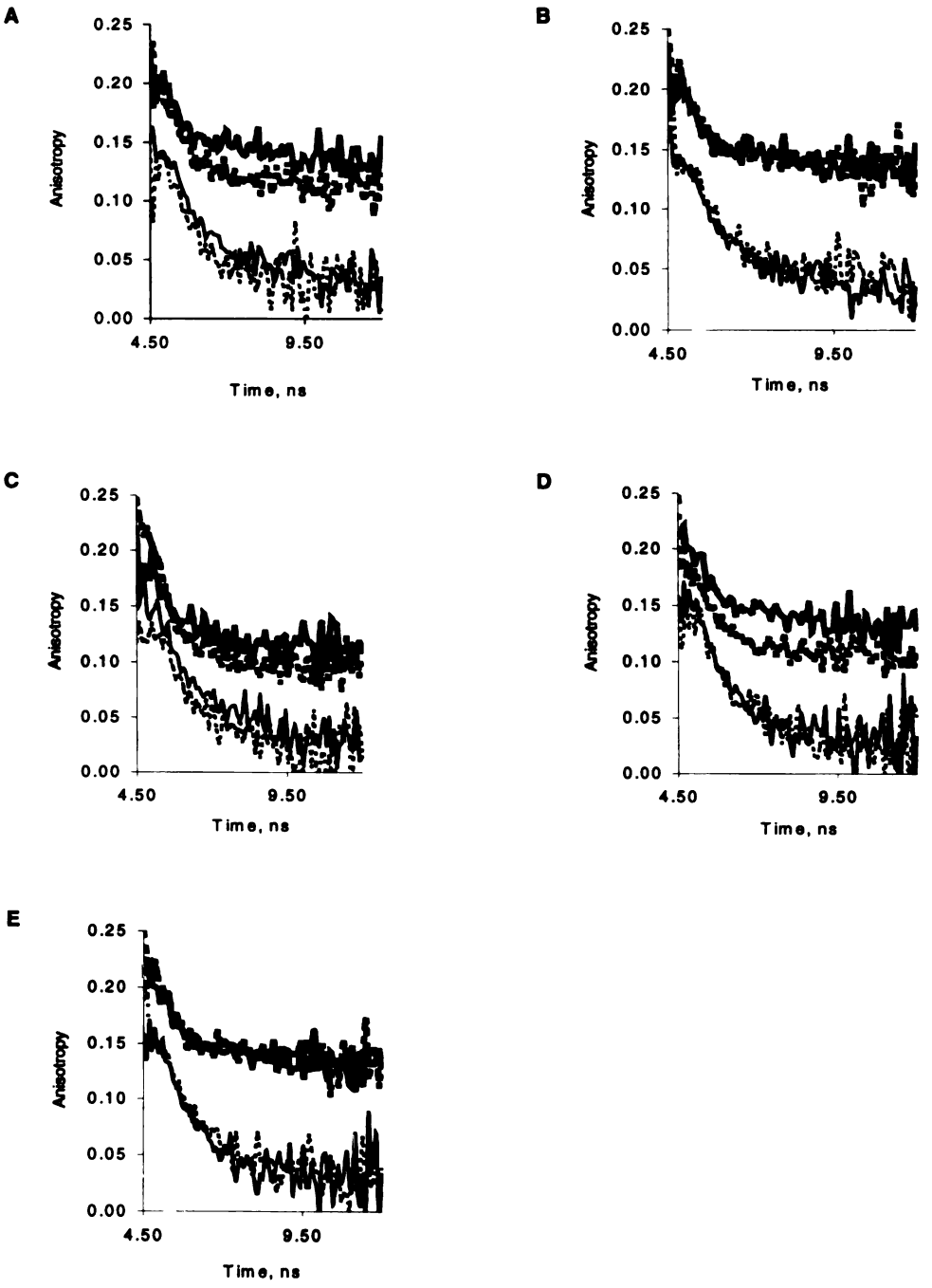

Figure 5.

overlap or nearly overlap those of the probe only protein (Figure 5A-E, heavy dotted and solid lines).

To quantify these results, best fit curves were generated (Table III). The anisotropy decays of all Gal4-VP16 proteins in the absence or presence of TBP were best fit to a two component model with a subnanosecond fast rotational correlation time, ϕ_1, representing segmental motion around the 5-hydroxytyptophan fluorophore and a slower rotational correlation component, ϕ_2, representing a more global motion of the protein. Rigorous confidence limits were not assessed for all parameters. The acceptable variation in the fitted rotational correlation times was assessed by fitting anisotropy decay data to fixed rotational correlation values.

The Gal4-VP16 proteins in the absence of TBP fit to a range of rotational correlation times, with ϕ_1 from 0.13-0.77 nanoseconds and ϕ_2 from 2.6-14 ns (Table III, top). However, the anisotropy decay data from the proteins bearing activation mutations also could be fit to the rotational correlation components of the proteins bearing the respective fluorophore alone (Table IV, top). In no case was the χ^2 value altered by more than 10%, and for all of the proteins with alanine substitutions except F475W/F479A, the change in χ^2 was less than 3.5%. Thus the variations between activation mutant and wild type protein are not

Table III. Fluorescence anisotropy decay parameters of the Gal4-VP16 proteins labeled with 5-OH-W in the absence and presence of TBP. Anisotropy decay curves were recorded for Gal4-VP16 proteins containing 5-OH-W and various alanine substitutions in the presence and absence of a 2-fold molar excess of TBP. Anisotropy decay data were analyzed by the "sum and difference" method (10). The rotational correlation times (ϕ) and the corresponding preexponentials (β) were derived from the best fit. The apparent limiting time zero anisotropy (r_o(app)) is defined as $\Sigma\beta_j$. r_o(app) was used to calculate the cone semiangles (Θ). The χ^2 value is calculated as a measure of the goodness of fit of the anisotropy decay curves and parameters.

Table III.

	β_1	ϕ_1	β_2	ϕ_2	ro (app)	Θ	χ^2
Without TBP							
442W	0.086	0.43	0.072	5.6	0.158	40.1	1.11
442W/444A	0.106	0.50	0.041	12	0.148	49.9	1.13
442W/475A	0.097	0.62	0.057	13	0.154	44.6	1.05
473W	0.081	0.29	0.102	3.3	0.183	34.8	1.02
473W/475A	0.117	0.26	0.084	2.6	0.200	42.1	1.11
475W	0.111	0.77	0.039	14	0.149	51.3	0.81
475W/479A	0.205	0.13	0.094	2.7	0.299	47.9	0.97
442A/475W	0.090	0.70	0.058	6.7	0.147	43.5	1.00
With TBP							
442W	0.060	0.18	0.154	36	0.213	26.4	1.02
442W/444A	0.053	0.47	0.123	53	0.175	27.5	0.88
442W/475A	0.057	0.35	0.133	50	0.190	27.4	1.03
473W	0.046	0.49	0.129	26	0.175	25.4	1.08
473W/475A	0.105	0.36	0.109	31	0.213	37.3	1.10
475W	0.074	0.16	0.154	25	0.228	28.8	0.95
475W/479A	0.056	0.49	0.115	48	0.171	28.9	1.01
442A/475W	0.054	0.22	0.151	37	0.205	25.5	1.07

Table IV. Fluorescence anisotropy decay parameters of the Gal4-VP16 proteins labeled with 5-OH-W in the absence and presence of TBP calculated with fixed rotational correlation times. Anisotropy decay curves were recorded for Gal4-VP16 proteins containing 5-OH-W and various alanine substitutions in the presence and absence of a 2-fold molar excess of TBP. Anisotropy decay data were analyzed by the "sum and difference" method (10). Parameters were generated by fixing both ϕ values to that of the appropriate probe alone protein. The χ^2 value is calculated as a measure of the goodness of fit of the anisotropy decay curves and parameters. Differences in χ^2 values between the parameters generated by initial calculations with free ϕ values (Table III) were calculated ($\Delta\chi^2$). The apparent limiting time zero anisotropy (r_o(app)) is defined as $\Sigma\beta_j$. r_o(app) was used to calculate the cone semiangles (Θ).

Table IV.

	β1	ϕ1	β2	ϕ2	ro (app)	Θ	χ2	Δ χ2
Without TBP								
442W	0.086	0.43	0.072	5.6	0.158	40.1	1.11	
442W/444A	0.099	0.43	0.052	5.6	0.151	46.1	1.10	2.7%
442W/475A	0.083	0.43	0.077	5.6	0.160	38.8	1.09	3.3%
473W	0.081	0.29	0.102	3.3	0.183	34.8	1.02	
473W/475A	0.127	0.29	0.074	3.3	0.200	44.8	1.09	1.3%
475W	0.111	0.77	0.039	14	0.149	51.3	0.81	
475W/479A	0.113	0.77	0.036	14	0.149	52.3	1.08	9.6%
442A/475W	0.103	0.77	0.045	14	0.149	48.3	0.98	1.7%
With TBP								
442W	0.060	0.18	0.154	36	0.213	26.4	1.02	
442W/444A	0.102	0.18	0.129	36	0.230	34.8	0.92	4.5%
442W/475A	0.091	0.18	0.138	36	0.229	32.6	1.07	4.2%
473W	0.046	0.49	0.129	26	0.175	25.4	1.08	
473W/475A	0.082	0.49	0.109	26	0.191	34.3	1.14	3.3%
475W	0.074	0.16	0.154	25	0.228	28.8	0.95	
475W/479A	0.103	0.16	0.125	25	0.228	35.3	1.16	12.9%
442A/475W	0.049	0.16	0.157	25	0.206	24.1	1.16	8.0%

considered significant and these proteins can be said to be indistinguishable.

In the presence of a twofold molar excess of TBP, the slow rotational correlation time, ϕ_2, increased for all proteins tested (Table III, bottom). The range of ϕ_2 values obtained was 26-53 ns. These rotational correlation times describe the motion of a protein larger than Gal4-VP16 alone, indicating the presence of a complex formed between Gal4-VP16 and TBP. Moreover, all proteins tested show a decrease in the contribution of the fast anisotropy decay component to the total anisotropy and a corresponding decrease in the cone semiangle, Θ. The fast anisotropy decay component, ϕ_1, reflects the segmental motion around the 5-hydroxytryptophan fluorophore. Its contribution to the overall anisotropy is given by β_1. If segmental motion of a fluorophore is modeled as the a wobbling of its transition moment within a cone, the extent of this motion is given by the cone semiangle magnitude (29, 42). Taken together, then, decreases in these two parameters in the presence of TBP indicate a decrease in segmental mobility of the VP16 activation domain in the presence of TBP for all of the proteins tested. Data obtained in the presence of TBP could not be fit to ϕ_1 and ϕ_2 values obtained from experiments in the absence of TBP, nor vice versa, demonstrating that the difference between the data sets is significant (data not shown).

In the presence as in the absence of TBP, the anisotropy decay parameters for proteins with a 442 or 473 fluorophore do not vary significantly between activation mutant proteins and their respective wild types. All three alanine mutant proteins – 442W/444A, 442W/475A, 473W/475A - can be fit to the ϕ_1 and ϕ_2 values from their respective fluorophore only proteins without altering χ^2 values by more than 4.5% (Table IV, bottom). Thus, although the increase in ϕ_2 indicates that a complex is formed between VP16 and TBP, these results indicate no effect of activation mutations on this interaction.

The proteins with a 475 fluorophore yield a slightly different result. The 442A mutation does not appear to have a significant effect. Anisotropy decay parameters obtained using the 442A/475W protein can be fit to the ϕ values of the 475 probe alone with only an 8% difference in χ^2. The 479A mutant, however, shows a change of 12.9% when its data are fit to the fixed rotational correlation parameters of the 475 probe alone. This is slightly larger than the 10% limit of significance and may indicate an effect of this mutation on TBP binding. This effect is slight, however, and when compared to the average value for the three 475 probe proteins, the 479A mutant χ^2 is altered by only 0.8% from the wild type fitting (data not shown). Moreover, this mutation does not affect the overall binding of Gal4-VP16 and TBP, as demonstrated by the K_D values calculated from the steady state data (Table II).

Table V contains values averaged from data for all proteins bearing the 5-hydroxytryptophan probe at the same position. When data for each protein are fit to these averaged ϕ_1 and ϕ_2 values, no χ^2 value changes by more than 8% (data not shown), indicating that variations among proteins bearing the same fluorophore are not significant. Therefore, the average values are representative of the behavior of a probe at a given position.

Upon addition of TBP, the ϕ_2 value increases and the β_1 and Θ values decrease for all proteins (Table V). However, the changes for the 473 proteins are not as large as for the 442 and 475 probes (compare data, Table V). The ϕ_2 correlation time increases 9.5 fold for the 442 and 475 proteins upon addition of TBP. For the 473 probe proteins, this increase is only 4.5 fold. Likewise, the decrease in β_1 and Θ values for the 442 and 475 proteins is approximately 40%, whereas for the 473 proteins these values only decrease by approximately 20%. The lower β_1 and Θ values indicate that the 473 position is less restricted in its segmental motion than the 442 or 475 positions. In addition, the ϕ_1 value increases for 473 and not for 442 or 475. This increase indicates that the apparent size of the segment associated with position 473 becomes larger in the presence of TBP. These results agree with those previously reported (53), and seem to indicate that different regions of VP16C interact differently with TBP.

Table V. Fluorescence anisotropy decay parameters of the Gal4-VP16 proteins labeled with 5-hydroxytryptophan in the absence and presence of TBP, averaged for proteins bearing the same probe. Anisotropy decay curves were recorded for Gal4-VP16 proteins containing 5-hydroxytryptophan and various alanine substitutions in the presence and absence of a 2-fold molar excess of TBP. Anisotropy decay data were analyzed by the "sum and difference" method (10). Parameters were generated for each protein separately (Table III), then average β_1, β_2, ϕ_1, and ϕ_2 values were calculated. The apparent limiting time zero anisotropy (r_o(app)) is defined as $\Sigma\beta_j$. ro(app) was used to calculate the cone semiangles (Θ).

	β1	φ1	β2	φ2	ro (app)	Θ
Without TBP						
442W	0.096	0.52	0.057	10	0.153	44.5
473W	0.099	0.27	0.093	3.0	0.192	38.6
475W	0.135	0.53	0.064	7.9	0.199	47.4
With TBP						
442W	0.057	0.33	0.137	46	0.194	27.2
473W	0.076	0.43	0.119	29	0.195	32.2
475W	0.061	0.29	0.140	37	0.201	27.7

Time resolved and steady-state anisotropy to determine the effects of TATA-box DNA on the TBP:VP16 complex. Time resolved anisotropy decay studies were carried out on a preformed complex of 2 μM Gal4-VP16 bearing a 5-OH-Trp fluorophore at position 442 and 4 μM TBP. After an initial anisotropy decay was measured, TATA-box DNA was titrated into the mixture and a new anisotropy decay reading was taken upon each addition of TATA-box DNA. The results of this experiment (Table VI) indicate that TATA-box DNA disrupted the binding of TBP to VP16. Increasing amounts of TATA-box DNA resulted in decreasing rotational correlation times, indicating a decrease in the size of the fluorophore complex. In the presence of a sufficient amount of TATA-box DNA, the ϕ_2 value dropped to nearly equal that of Gal4-VP16 in the absence of TBP. Moreover, the decrease in ϕ_2 occurred in a dose-dependent manner.

These results must be considered suspect as the TATA-box DNA used in this experiment was annealed improperly. Instead of being heated to 90°C and allowed to cool together to room temperature, the complimentary single stranded oligonucleotides were resuspended in approximately 25 μl of TE and incubated at room temperature for almost 30 minutes. Thus, the degree of hybridization of the complementary oligonucleotides is uncertain. The effect observed on anisotropy of the TBP:VP16 complex may have been due to molecular crowding by single stranded DNA preventing reformation of TBP:VP16 complexes following

Table VI. Time resolved anisotropy of Gal4-VP16/TBP complex upon addition of TATA-box DNA. A pre-formed complex of 2 mM Gal4-VP16 F442W and 4 mM TBP was used in this experiment. "W" denotes 5-OH-tryptophan. Anisotropy decay data were analyzed by the "sum and difference" method (10). The long rotational correlation time (ϕ_2) was derived from the best fit.

[TATA-box DNA]	ϕ_2
Gal4-VP16 only	7.1 ns
0 μM	45 ns
0.98 μM	46 ns
1.92 μM	40 ns
3.70 μM	18 ns
6.90 μM	9.6 ns

equilibrium dissociation. Specific or non-specific binding to single stranded DNA may also have produced this result.

To address this issue, steady state anisotropy experiments were performed with double stranded TATA-box DNA that was properly annealed. Results of this experiment are shown in Figure 6. The addition of TATA-box DNA did decrease anisotropy values in a dose-dependent manner, indicating that the TBP:VP16 complex was being disrupted (Figure 6, diamonds). However, addition of TATA-box DNA in this experiment did not reduce anisotropy to the value seen with Gal4-VP16 alone (Figure 6, circle). Moreover, addition of equimolar amounts of either single stranded oligonucleotide also resulted in a decrease in anisotropy (Figure 6, square and triangle). While the addition of the maximum amount of TATA-box DNA decreased anisotropy by 71%, the addition of an equivalent amount of ST274 decreased anisotropy 52% while ST275 decreased it by 31%. The difference between the effects of the double stranded TATA-box DNA and the single stranded ST274 on anisotropy values is the same as the difference between effects of ST274 and ST275 on anisotropy values. This effect of single stranded DNA makes interpretation of these results problematic. If the oligonucleotides used in the time resolved experiment were not completely annealed, then the molar amount of DNA added could have been as much as twice that of the single and double stranded DNA used in the steady state

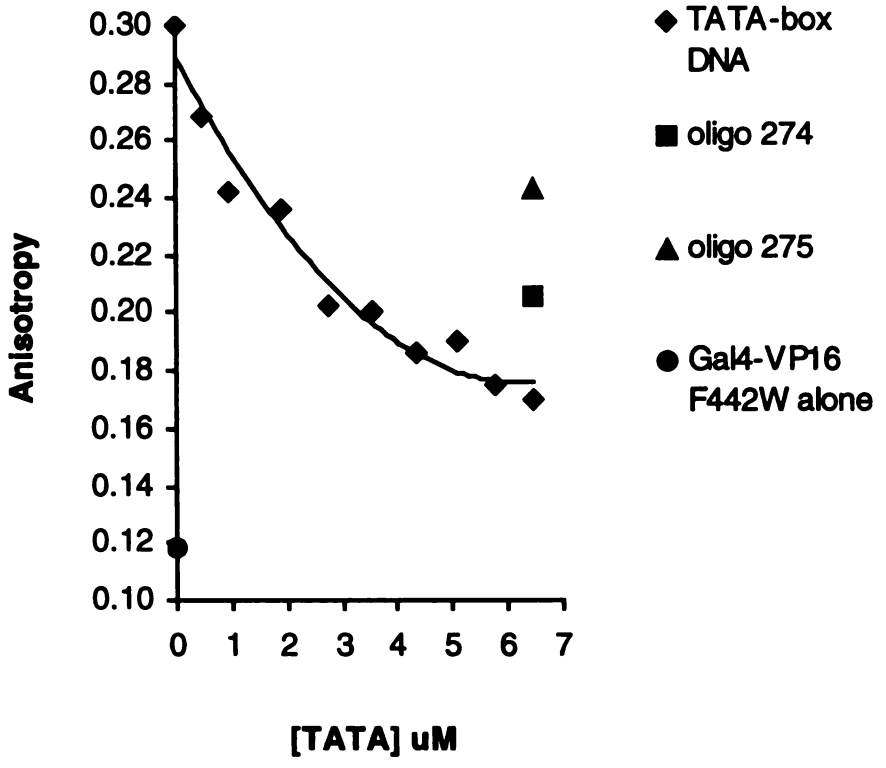

Figure 6. Steady state anisotropy of Gal4-VP16:TBP complex upon addition of TATA-box DNA. A pre-formed complex of 2 μM Gal4-VP16 F442W and 4 μM TBP was used in this experiment. Diamonds with the solid line represent anisotropy of Gal4-VP16 F442W upon titration of TATA-box DNA. The square and the circle represent anisotropy of Gal4-VP16 F442W upon addition of each of the single stranded oligos used to create the TATA-box. The circle represents the anisotropy value for Gal4-VP16 F442W in the absence of TBP and DNA. "W" denotes 5-OH-tryptophan.

experiment. It seems likely that there is at least some effect of single stranded DNA.

DISCUSSION

Fluorescence anisotropy and the TBP:VP16 interaction

Most proposed models for the function of eukaryotic transcriptional activators invoke contact between the activator and some target in the transcriptional machinery. Many transcriptional activators bind *in vitro* to a number of putative target proteins. VP16, for example, has been shown to bind to TFIIA (31), TBP (55), $dTAF_{II}40$ or $hTAF_{II}32$ (15, 30), TFIIB (41), TFIIH (64), the mediator in a PolII holoenzyme (19), and the yeast adaptor ADA2 (3, 54). An important yet unresolved question is which if any of these interactions are relevant to transcriptional activation *in vivo*.

Mutational analysis of the VP16 activation domain has identified amino acid residues, especially Phe442, Phe473, Phe475, and Phe479, that are critical for activation ability (9, 48, 56). Affinity chromatography experiments have demonstrated that mutations at position 442 that disrupt activation by VP16N also disrupt binding to TBP (17, 22). Immunoprecipitation experiments have shown that mutations at positions 473 and 473 together, or at position 479 alone also diminish binding to TBP (32). To explore these correlations simultaneously in the

context of the full length activation domain, we chose to employ fluorescence spectroscopy.

Unlike binding assays that give a result for the protein in its entirety, fluorescence spectroscopy with an intrinsic fluorophore allows one to look directly at the position in which the fluorophore is placed. Moreover, fluorescence, by providing information about the microenvironment surrounding the fluorophore residue, is sensitive to the nature of the interaction in a manner in which binding assays are not (36). The ability to place fluorophores at different positions within the protein allows one to study the affects of an interaction on different segments of the protein. For example, such a strategy allows study of how mutations in one domain of a protein affect the structure of another. Furthermore, fluorescence anisotropy experiments allow determination of dissociation constants and a view of the global motion of the protein, thereby also providing information about the protein as a whole. For these reasons, we chose to utilize fluorescence spectroscopy in this study.

Results of steady-state anisotropy experiments indicated little or no effect of transcriptionally debilitating mutations in VP16 on the association between the VP16 activation domain and TBP. For all proteins tested the calculated K_D values were equivalent for proteins bearing the 5-OH-tryptophan fluorophore alone or the fluorophore with a

transcriptional mutation. However, proteins with fluorophores at position 475 in VP16C demonstrate higher K_D values than those with fluorophores at the 442 or 473 positions. This seems to indicate that the differences result from the insertion of the tryptophan analogue itself at a given position. The K_D values determined here for the 442 and 473 fluorophores are on the order of 4×10^{-7} M and 6×10^{-7} M respectively, which agree with a previously reported K_D value of 2×10^{-7} M for the interaction of VP16 and TBP (22). The calculated K_D for the 475 fluorophore is on the order of 1×10^{-6} M. Although no explanation is available for the discrepancy between K_D values for a fluorophore at position 473, there is a tendency toward higher K_D values when the fluorophore is placed in the VP16C subdomain as opposed to VP16N. The substitution of the fluorophore at these transcriptionally critical positions in VP16C affects the binding of VP16 and TBP, suggesting that despite the lack of effect of the alanine mutations at these positions on observed fluorescence, the transcriptionally critical amino acid residues for which the fluorophores were substituted may affect the interaction of VP16 and TBP.

Emission spectra and time resolved anisotropy decay spectra recorded for the Gal4-VP16 proteins in the absence of TBP indicate that fluorophores at all three positions (442, 473, 475) are solvent exposed and highly mobile. Time resolved anisotropy decay data indicate that the

addition of TBP restricts the segmental mobility of all fluorophores in VP16. The presence of TBP in these experiments caused an increase in the slow anisotropy decay component and a decrease in the contribution of the fast anisotropy decay component. These characteristics indicate that the VP16 activation domain becomes structurally constrained in the presence of TBP.

For the majority of the Gal4-VP16 proteins in the presence of TBP, those bearing activation mutations can be fit to the parameters of their respective fluorophore proteins, indicating no significant difference in the VP16:TBP interaction as a result of these mutations. Moreover, the data from each protein carrying a given fluorophore can be fit to the average data for the set of proteins carrying that fluorophore. The results of this analysis indicate that the 442 and 475 fluorophores behave similarly and somewhat differently from the 473 fluorophore. As with the steady state K_D values, this suggests that the presence of the fluorophore at a given position exerts an effect on TBP interaction. Transcriptional assays indicate that residues 442 and 475 are more important than residue 473 for transcriptional activation ability (9, 48, 56), a result which correlates with the time resolved data. In contrast, however, the K_D values indicate a greater similarity between positions 442 and 473. Taken all together, these results suggests that while some connection between TBP

interaction and transcriptional activation may exist, other contributing factors must exist.

In the proteins tested, only the presence of the 479A mutation has even a minimal effect on TBP interaction in time resolved anisotropy experiments. This is consistent with the observation that the F479A mutation modestly decreases the ability of VP16C to bind TBP *in vitro* (32). However, the anisotropy decay parameters obtained for the 475W/479A protein can be fit to the average of the fitted parameters for all proteins with a 475 fluorophore, which indicates that the differences are likely not significant. Although these results direct attention to position 479, neither the time resolved anisotropy data nor the *in vitro* binding experiments (32) demonstrate a strong effect of the 479A mutation on TBP interaction.

The difference in anisotropy data for the 475W/479A protein could be explained by an alteration in the nature of the fluorophore itself. In addition to segmental and global motion of the fluorophore containing protein, the vibration or rotation of the probe on its own axis can affect its fluorescence (36). If the amino acid residues at positions 475 and 479 are adjacent in the secondary structure of the protein, each of these residues would potentially sterically affect the other. Uesugi and coworkers observed that $hTAF_{II}32$ induces formation of an alpha helix between residues 472 –483 VP16C in which Phe 475 and Phe479 are

adjacent to one another. The simplest hypothesis supposes that the target-induced structure in VP16 is characteristic of VP16 and would be the same regardless of the target. Thus, a 5-OH-tryptophan at position 475 might be expected to vibrate or rotate far more freely in the presence of a small neighboring residue like alanine than the much larger phenylalanine, contributing to more rapid rotational correlation times. In fact, the rotational correlation times for the 475W/479A protein in the absence of TBP are shorter than those of the other proteins that carry the 475 fluorophore. This proposed steric difference could also account for the change in fluorescence intensity observed for this protein in the steady state anisotropy experiments.

Although the results of these experiments indicate that none of the alanine mutations have an effect on VP16:TBP interaction, both the steady state and time resolved anisotropy data sets confirm an interaction between the VP16 activation domain and TBP. However, unlike the majority of the binding and activity assays, which have tested either VP16N or VP16C separately, these experiments have been conducted with the full length activation domain. Previous fluorescence experiments showed no difference between the behavior of the 442 fluorophore alone in the presence of TBP in the VP16N or full length context (53). However, activity assays have shown that the activity of a transcriptionally defective VP16N mutants can be partially restored by

the addition of the VP16C subdomain (48). It is possible, therefore, that the slight nature of the effects seen here of mutations that in an isolated subdomain have had more severe effects on either binding or activation may be due to compensation by the other subdomain. Moreover, *in vivo* transfection experiments have recently demonstrated that VP16N does not recruit TBP to the TATA box in mammalian cells *in vivo* (18). Therefore, although both subdomains of VP16 activation domain can bind to TBP *in vitro*, it is possible that the relevant interaction with TBP *in vivo* is strictly a function of either the VP16C or the VP16N subdomain. In that case, the presence of the other subdomain in one-on-one binding assay of the type used here may compensate for lack of binding in a manner which does not occur *in vivo*. Further study of these mutations in the context of the isolated VP16N and VP16C subdomains is needed to explore these questions.

TBP is not the only component of the transcriptional machinery which has been associated with activation by VP16. Binding of the p62 subunit of TFIIH to VP16C has been shown to correlate directly with activation ability (32, 64). Both binding to TFIIA and the formation of a TFIIA:TFIID:promoter DNA complex also correlate to activation ability with a set of VP16C mutants (32). Certain residues implicated in binding of VP16C to $TAF_{II}32$ have been shown to be important for activation, although the correlation does not hold for all mutants tested (56, 61;

Y.Nedyalkov, S.M.S., P.J.Horn, and S.J.Triezenberg, unpublished). Given the number of interactions that might contribute to transcriptional activity, it is perhaps naïve to expect to find a correlation between transcriptional activity overall and any one target.

It is also possible that VP16 acts at multiple steps in transcriptional activation, either both subdomains in concert or separately. Moreover, mutations in TBP that decrease transcriptional activation by VP16 can be found at the TFIIA and TFIIB binding interfaces of TBP (5, 26, 37, 59), suggesting that VP16 may not bind directly to TBP but might associate with it through another protein. If the interaction with TBP is relevant to a different step than is affected by the transcriptional mutations tested, or if the interaction *in vivo* is not direct as it is *in vitro*, no correlation would be found. Fluorescence studies with other potential targets for VP16 might discover a target on whose binding these mutations have a direct effect.

Association of TBP with VP16 and TATA-box DNA

TBP derives its name, TATA-box binding protein, from its ability to bind the TATA-box DNA. X-ray crystal structures for TBP bound to DNA have been solved (25, 28). Chemical crosslinking and affinity chromatography suggest that both TBP and TFIID exist as a dimer in solution, raising the possibility that the function of transcriptional activators with respect to TBP is to dissociate the TBP dimer thus

allowing TBP to interact with TATA-box DNA (8, 57). Moreover, a mutation in TBP that disrupts transcriptional activation by VP16 can be found on the DNA binding surface of TBP (26). In order to explore the nature of the interactions among VP16, TBP, and DNA, we made use of fluorescence anisotropy to determine whether a TBP-VP16 complex could be dissociated by addition of TATA-box DNA.

Results of the preliminary experiments described herein suggest that TBP cannot bind both VP16 and DNA simultaneously. These observations are intriguing but lack certain information which would make them definitive. Titration of the single stranded DNA controls and a non-specific double stranded DNA under the conditions of the experiments conducted here would provide a direct comparison of the dose dependent effect of DNA on TBP-VP16 binding and would allow determination of the specificity of the effect. Use of fluorescently labeled DNA would address this issue more directly, as it would be possible to determine whether upon dissociation from VP16 the TBP binds the TATA-box DNA (through observation of an increase in the anisotropy of the DNA) or remains in solution. The reverse experiment, using prebound TBP:TATA and titrating VP16, would also be useful in analyzing the specificity of this effect. Overall, these experiments provide an intriguing preliminary observation, but no hard evidence of mutually exclusive interactions between TBP and VP16, and TBP and DNA.

Summary and Conclusion

Clearly the binding of VP16 and TBP, and the restriction of motion of the VP16 activation domain by TBP demonstrated in these experiments is real and specific *in vitro*. Previous work has demonstrated that another transcription factor, TFIIB, which can bind to VP16 cannot induce this conformational constraint (53). Moreover, preliminary experiments with TATA-box DNA suggest that specific binding of TBP and TATA may be related to the interaction between TBP and VP16.

The results of this study, however, also confirm that the binding of the VP16 activation domain to TBP is not the sole determinant of transcriptional activation ability *in vivo*. However, studies of this type attempt to look at simple pieces of a very complex system. *In vivo* transcriptional activation will not be completely understood until there is a way in which to study it directly. In the meantime, however, the results presented here confirm the value of fluorescence spectroscopy as a tool to study protein-protein interaction. Future experiments of this type may well allow the identification of *in vivo* relevant binding partners for VP16 activation domain as well as exploring how those interactions affect the N and C subdomains thereof.

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CHAPTER 6

SUMMARY AND FUTURE STUDY

SUMMARY

The focus of my thesis research was the transcriptional activation domain of the herpes simplex virus transactivator VP16. Using oligonucleotide directed mutagenesis and *in vivo* activity assays, I conducted a thorough mutational analysis of the C-terminal subdomain of the VP16 activation domain (VP16C). Analysis of VP16N had established the hypothesis that bulky hydrophobic amino acids are critical for transcriptional activation regardless of which amino acid residues are most abundant in an activation domain (5, 29). This theory has subsequently been supported by work on numerous other activation domains (1, 3, 9, 12, 14, 21, 22, 31). The conclusions of this study of VP16C clearly indicate that VP16C also follows this pattern. The amino acid residues most critical for activation by VP16C in yeast or in mammalian cell assays are phenylalanines at positions 473, 475, and 479, and glutamate 476. Substitution of any bulky hydrophobic amino acid at position 473, 475, or 479 resulted in wild type or near wild type transcriptional activity, whereas other substitutions decreased function. At position 476, only substitution to alanine greatly diminished

transcriptional activation suggesting a steric role for this side chain that alanine is too small to fulfill.

Having completed the mutational characterization of the VP16 activation domain, I sought to use the VP16 mutants to investigate the link between *in vitro* binding and *in vivo* activation. The activation domain of VP16 has been shown to bind to multiple potential target proteins (10, 17, 18, 23, 38), including the yeast adaptor Ada2 (2, 33). To analyze the effects of mutations in VP16C on binding to Ada2, I made use of reporter gene assays in a *Δada2* yeast strain. The results of these experiments indicate that VP16C and Ada2 do not directly interact *in vivo*.

VP16 has also been shown to bind the basal transcription factor TBP (11, 35). From mutational analysis, I and others found that transcriptionally critical phenylalanine residues in the activation domain of VP16 can be replaced by tryptophan with no significant effect on activation (5, 29; Chapter 3, this work). Tryptophan is a natural fluorophore; moreover, the excitation spectrum of the 5-hydroxytryptophan analogue is shifted to longer wavelengths than that of native tryptophan, allowing selective excitation of 5-hydroxytryptophan in the presence of normal Trp residues. Based on the results of the mutational analysis, I was able to place unique intrinsic probes for

fluorescence spectroscopy at those positions in VP16 that are most critical for *in vivo* transcriptional activation.

Using fluorescence anisotropy techniques, I analyzed the interaction between VP16 and TBP. 5-hydroxytryptophan probes were placed at transcriptionally critical positions in both the N and C subdomains (positions 442, 473, and 475). Proteins bearing these probes alone, or these probes in combination with alanine mutations known to disrupt activation were studied in the presence and absence of TBP. In the presence of TBP, the highly mobile VP16 activation domain becomes more constrained, indicative of formation of ordered secondary structure. However, the same TBP-induced structure in VP16 is formed in the presence or absence of transcriptional mutations. These results indicate no correlation between these transcriptional activation mutants and binding to TBP for the VP16 activation domain.

The experimental approaches outlined above highlight the true value of the thorough mutagenic analysis described in this thesis. Knowing which mutations disrupt transcription *in vivo* provides clues as to which parts of the protein to study. A more subtle benefit, however, is found in knowing which changes do not disrupt activity. Biophysical techniques such as fluorescence that require the use of amino acid analogues depend on a thorough mutational analysis to delineate the limits within which changes can be made in VP16 activation domain

while allowing the protein to remain representative of the wild type. An extensive mutational analysis such as has now been completed on both subdomains of VP16 is vital as a foundation on which to build functional experiments which are representative of wild type *in vivo* activation.

In the process of conducting these studies, I also made improvements to two of the procedures utilized by our laboratory. The manner in which yeast cells are grown for assay was altered and the reproducibility of β-galactosidase assays was increased. Previously, individual yeast colonies had been selected and grown in liquid culture overnight before assay. When activities from three separate cultures were averaged, standard deviations were frequently as high as half the activity value. In an effort to produce more replicable results, I altered both colony selection and cell growth procedures. To assay a representative population of yeast, I replaced the individual transformants with a pool of approximately 100 colonies. To ensure that all cultures were at the same growth phase and approximate cell density, I diluted overnight cultures to OD_{600} of 0.1 and harvested them at OD_{600} of 0.5. These changes in procedure resulted in a decrease in standard deviation from as much as 50% to 10% or less. The new method for cell growth also allowed activity assays and Western blot analysis to be performed on extracts from cells grown in the same culture, which

eliminates the possibility of differences in growth conditions between assays.

Significant changes were also made to the procedure for purifying Gal4-VP16 fusion proteins. By altering the concentrations of both PEI and ammonium sulfate in precipitation steps, and employing HPLC as opposed to conventional chromatography techniques, I eliminated two dialysis steps and one column chromatography step. The new procedure is two days faster and yields twice as much protein as the previous method (32). The protein produced was as pure or purer than that produced by the old method, as assessed by Coomassie Blue staining of SDS-PAGE gels.

FUTURE STUDY

The process of transcriptional activation requires DNA and multiple proteins in addition to a transcriptional activator (37). The most sophisticated X-ray crystal structures of this multiprotein-DNA complex available to date are binary complexes between TBP and DNA (15, 16), and ternary complexes with TFIIA or TFIIB in addition (8, 25). Photocrosslinking has allowed visualization of larger complexes, but only through the use of protein-DNA cross-linking agents such that protein-protein associations are not mapped (19, 30). An extensive panel of alanine scanning mutants in TBP allowed a simultaneous view of TBP,

PolII, TFIIA, TFIIB, and TFIIF (36); however, the binding experiments were performed singly between TBP and each binding partner. Binding experiments of this type negate any effects of other members of the complex on a given interaction.

The fluorescence methods which have been employed in this thesis and previously in the lab in collaboration with the Knutson group have great potential structural studies of the entire transcription complex. The availability of tryptophan analogues with fluorescence properties different from those of native tryptophan allow observation of a single protein in the presence of multiple others (20). Fluorescent amino acid analogues such as 7-azatryptophan and 5-hydroxytryptophan, which can be selectively excited in the presence of native tryptophan, and 4-fluorotryptophan, which does not absorb at the wavelengths which excite the fluorescent tryptophans, can be used to "customize" the transcriptional system. Using combinations of proteins containing native tryptophan, proteins synthesized in the presence of various tryptophan analogues, and fluorescently labeled DNA, individual components can be selectively observed in the context of an entire transcriptional complex. Experiments of this sort would provide information as to structural changes in a protein of interest, for example VP16, in the presence of multiple targets. Moreover, resonance energy transfer techniques, which provide information about the proximity of two fluorophores in a mixture

(20), can be used to investigate which target protein is responsible for a given structural change. We have begun to apply this approach in the analysis of VP16-TBP-TATA interactions described in this thesis. These studies should be pursued and expanded to provide a clearer picture of the behavior of VP16 in the context of the transcription complex.

An issue that must be considered in analyzing the results of these studies is the possibility of a temporal component of transcriptional activation. For example, it is possible that the interaction of VP16 with a target protein is important at more than one step in transcriptional activation, such that VP16 acts at multiple steps in transcription through one or more target protein. The ability of VP16 to interact with TBP, for example, when viewed via fluorescence spectroscopy does not correlate to the effects of mutations at amino acid residues in VP16 that are critical for transcriptional activation. However, TBP binds to both VP16N and VP16C and induces an ordering of the structure of both activation subdomains. TBP binding may be an artifact of a "sticky" VP16 activation domain which is capable of binding to many proteins in an *in vitro* system; however, it is equally possible that the TBP interaction is important, but at a step later than that arrested by FA442, LA444, FA475, or FA479 mutations.

To address this issue requires the ability to separate the steps of transcriptional activation. Work from a number of laboratories has

elucidated the sequential assembly on promoter DNA of the preinitiation complex (4, 6, 7, 13, 24, 27). If VP16 is involved in multiple steps in transcriptional activation, anisotropy measurements taken at each assembly step would record the binding, dissociation, and rebinding of VP16 to target proteins upon sequential addition of transcription factors. Recently, stopped-flow capability has become available for the fluorescence spectrophotometers of the Knutson group. In combination with the use of tryptophan analogues to provide a unique fluorophore, this would allow the VP16 activation domain to be observed during the step-wise reconstitution of the transcriptional system.

Gal4-VP16 has been referred to as "the ubiquitous transcriptional activator." This description highlights the fact that the biological context of the VP16 protein is the activation of immediate early genes in infection by the herpes simplex virus (reviewed in 26). Little use has been made of VP16 mutations in the context of the herpes virus itself. Recently, two groups have published reports on the effects of disruption or deletion of the VP16 activation domain on the phenotype of herpes simplex (28, 34). Work from this laboratory has explored the effects of deletion of VP16N, VP16C, or the entire activation domain (R.Pichyangkura and S.J.Triezenberg, unpublished). A logical progression from the deletion mutagenesis would be the incorporation of site specific mutations into recombinant virus to study the mechanism of VP16 activation in the

virus itself. Mutations such as F442A and F475A, which disrupt transcriptional activity in artificial systems (5, 29; Chapter 3, this work), may or may not affect the viral function. The extensive panel of VP16 activation mutants available will allow as thorough a characterization of activation in the virus as has been completed for the activation domain alone (5, 29; J.Kastenmeyer, P.Horn, S.J.Triezenberg, unpublished; Chapter 3, this work).

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