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SITE-SPECIFIC AND RANDOM MUTAGENESIS STUDIES OF THE VP16 TRANSCRIPTIONAL ACTIVATION DOMAIN

By

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ABSTRACT

SITE-SPECIFIC AND RANDOM MUTAGENESIS STUDIES OF THE VP16 TRANSCRIPTIONAL ACTIVATION DOMAIN

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Transcriptional activator proteins are an integral part of the transcriptional regulatory apparatus. However, little is known about the structural features of transcriptional activation domains that are important for their function. We have examined structural features of the activation domain of VP16, a transcriptional activator protein from herpes simplex virus type 1, by oligonucleotide-directed and random mutagenesis methods.

Extensive mutagenesis at a phenylalanine residue at position 442 of a VP16 activation domain truncated after amino acid 456 demonstrated the importance of an aromatic amino acid at that position. Based on an alignment of the VP16 sequence surrounding Phe442 and the sequences of other transcriptional activation domains, leucine residues at positions 439 and 444 of VP16 were subjected to mutagenesis. Results from these experiments suggest that bulky hydrophobic residues flanking Phe442 also contribute to the function of the truncated VP16 activation domain. Restoration of aa 457-490 to various

truncated Phe442 mutants restored partial activity. Although a pattern of amino acids surrounding Phe473 resembles that surrounding Phe442, mutations of Phe473 (or Phe475) did not dramatically affect activity. We infer that the two regions of VP16 (aa 413-456 and 457-490) possess unique structural features, although neither appears consistent with two prior models (the amphipathic alpha-helix or "acid blob" models) purporting to describe the structure of acidic activation domains such as that of VP16. These results, considered with previous in vitro activation and inhibition studies, suggest that the two subdomains of VP16 affect transcription by different mechanisms.

To identify mutations that decrease the activity of the distal subdomain (aa 457-490), I employed random mutagenesis combined with a biological selection in yeast. This approach was based on the observation that the GAL4-VP16 fusion protein, when overexpressed in yeast, is toxic to the growth of the host cell; mutations decreasing the activity of the VP16 activation domain relieve this toxicity. Four mutations in the distal subdomain (TA458/DN461/MV478, TA480, IV485, and YC465) were identified that decreased the activity of that domain.

I praise You
because I am fearfully and
wonderfully made;
Your works are wonderful,
I know that full well.

Psalm 139:14

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LIST OF ABBREVIATIONS

A_{420}	absorbance at 420 nm
A_{595}	absorbance at 595 nm
A_{600}	absorbance at 600 nm
aa	amino acid
AAH	amphipathic alpha helix
ATP	adenosine triphosphate
bp	base pair
CTD	carboxyl terminal domain of the largest subunit of RNA pol II
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HSV-1	herpes simplex virus type 1
ICP	infected cell protein
IE	immediate early
kDa	kilodaltons
LTR	long terminal repeat
MSV	murine sarcoma virus
NTP	nucleotide triphosphate
ONPG	o-nitrophenyl-β-D-galactoside
PEG	polyethylene glycol
PMSF	phenylmethanesulfonyl fluoride
RNA pol II	RNA polymerase II
SDS	sodium dodecyl sulfate

LIST OF ABBREVIATIONS (cont'd)

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAF	TBP associated factor
TBP	TATA box-binding protein
TFII	transcription factor of RNA pol II
tk	thymidine kinase
UAS	upstream activating sequence
VP16	virion protein number 16

Single letter abbreviations for the amino acids: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

CHAPTER I

INTRODUCTION

Eukaryotic Transcription

The ability of a cell to regulate the expression of its genes is important, not only for its own viability but also for its appropriate function within the context of its environment. Genes contain the information for all cellular processes. In eukaryotes, the flow of genetic information from DNA to protein begins with the transcription of a gene into pre-messenger RNA, which after processing is exported from the nucleus to the cytoplasm where it is translated into protein. This multistep process of gene expression contains numerous points of regulation.

The state of the DNA template can affect the degree of expression of a particular gene. The methylation of cytosine residues can control gene expression, either positively or negatively, depending on the location of the methylated cytosines relative to the gene. In general, in higher eukaryotes genes which are constitutively expressed are undermethylated. An example of the repressive effects of methylated DNA is the murine α 1(I) collagen gene, in which methylation of a specific region downstream of the transcription startsite represses transcriptional activity (Rhodes and Breindl, 1992). A direct correlation between undermethylation and gene expression is not universal; for instance, there appears to be no correlation between the degree of methylation and the level of expression of the chicken lysozyme gene (Wölfl et al., 1991).

DNA usually exists in complex with histone proteins, forming structures called nucleosomes. Nucleosomes can have a repressive effect on gene transcription by physically excluding the binding of proteins necessary for transcription or blocking the movement of the enzymes involved in transcription. Nucleosome displacement likely involves multiple steps, including nucleosome destabilization followed by transfer of histone proteins onto competitor molecules (Adams and Workman, 1993). Another DNA state which has a role in regulating gene expression is the degree of supercoiling, which can increase the stored free energy of a region of DNA, thereby making it easier to unwind the DNA duplex.

The initiation of transcription is also a point of gene regulation. As will be described in greater detail below, a discrete set of proteins must assemble at a promoter for transcription to begin. In principle, factors that promote the binding or activity of one or more of these transcription proteins can enhance the rate of transcription initiation. Besides the assembly of transcription factors at the promoter, the initiation of transcription requires hydrolysis of ATP and the separation of the DNA strands; any factor that facilitates either of those events can also increase transcription initiation.

Once transcription has been initiated, the elongation of the nascent RNA transcript affords another opportunity for regulation. A good example of this is found in the human immunodeficiency viruses HIV-1 and HIV-2. Transcription occurs from the viral LTR promoter, but in the absence of the virus-encoded protein Tat little full-length viral RNA is produced. When Tat is present it not only stimulates transcription but, probably in combination with cellular factors, also facilitates efficient elongation complexes (Cullen, 1990). A second example is that of transcription factor IIS (TFIIS), which binds to RNA polymerase II and facilitates its movement past

intrinsic transcription pausing sites in vitro (Reinberg and Roeder, 1987b; Rappaport et al., 1988; Reines et al., 1989).

The diversity of proteins in a eukaryotic cell is determined to a large extent by alternative splicing of precursor messenger RNA, which allows synthesis of multiple proteins from a single gene. An excellent example of this is the α-tropomyosin gene. Tropomyosin proteins are essential components of the contractile apparatus in several different tissues, and each contractile cell type contains a specific tropomyosin that differs from that in other cell types. The diversity of tropomyosin proteins does not arise from multiple genes, but rather from differential splicing of the primary transcript from a single α-tropomyosin gene; at least seven different tropomyosin proteins are known (Breitbart et al., 1987).

The multiple poly(A) sites (designated L1-L5) of the adenovirus major late transcription unit (MLTU), a complex transcription unit encoding five proteins, exhibit different rates of processing depending upon the stage of infection of the virus. In an early stage of adenovirus infection, the 5' proximal L1 site is used almost exclusively even though transcription extends past the L2 and L3 sites. In later stages of infection, all five poly(A) sites are utilized efficiently (Prescott and Falck-Pedersen, 1992).

After processing in the nucleus, the mature mRNA is transported through the nuclear pore complex to the cytoplasm for translation. In the case of HIV-1 mRNAs, however, incompletely spliced forms of certain transcripts are transported to the cytoplasm, despite cellular mechanisms designed to prevent such events. The essential viral proteins Gag and Env are encoded by incompletely spliced HIV-1 mRNAs. The transport of these incompletely spliced transcripts is due to the action of the virus-encoded Rev protein, which binds specifically to a sequence present in the incompletely

spliced viral transcripts and allows their transport into the cytoplasm (Malim et al., 1989).

The prevalent model for the initiation of translation in eukaryotes is that the 40S ribosomal subunit binds to the 5' cap structure and migrates 5' to 3' along the mRNA until it encounters the first AUG codon; the complete ribosome initiates translation at that codon if it is in the proper sequence context (Kozak, 1991). However, in some instances alternative translation start site selection occurs. The cAMP-responsive-element modulator encodes both repressors and an activator of cAMP-responsive transcription. One of the repressor proteins results from the use of an internal AUG codon within the mRNA that also encodes the activator protein (Delmas et al., 1992).

Because of the inherent complexity of gene expression, those interested in the regulation of gene expression have many options for study. The research focus of our laboratory, as well as that of many others, is the regulation of gene expression at the level of transcriptional initiation of genes transcribed by RNA polymerase II.

Eukaryotic genes that encode proteins are transcribed by the multi-subunit RNA polymerase II enzyme (RNA pol II), whereas the genes encoding ribosomal RNAs and those encoding transfer RNAs (and the 5S ribosomal RNA) are transcribed by RNA polymerases I and III, respectively (Young, 1991). RNA pol II is comprised of 10 ± 2 subunits, depending upon the particular eukaryote (Young, 1991). The two largest subunits of RNA pol II are related to the β and β' subunits of prokaryote RNA polymerase, and are responsible primarily for catalysis of RNA chain elongation (Young, 1991). Additionally, three subunits of 14-28 kDa in RNA pol II are shared with the other two eukaryotic RNA polymerases (Young, 1991). The genes for all eleven subunits of S. cerevisiae RNA pol II have been cloned and their

proteins expressed and purified; two of these subunits, numbers four and seven, are not essential for mRNA synthesis in vivo, but together form a subcomplex that appears to influence the efficiency of transcription initiation (Young, 1991).

An especially interesting feature of the largest subunit of RNA pol II is a carboxyl terminal domain (CTD) consisting of multiple repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Young, 1991). This heptapeptide sequence is repeated 52 times in mammals, 44 times in Drosophila, and 27 times in yeast (Young, 1991). The CTD can be phosphorylated in vivo , and it is the major site of phosphorylation in RNA pol II (Young, 1991). RNA pol II binds to the promoter in the unphosphorylated state (denoted form IIa); however, phosphorylation of the CTD (generating form IIo) is required for the enzyme to clear the promoter and begin transcription (Cadena and Dahmus, 1987; Payne et al., 1989; Lu et al., 1991). Potential CTD kinases are discussed later in this chapter. In addition to functioning as a "trigger" mediating the transition of RNA Pol II from DNA binding to elongation (Peterson and Tjian, 1992), the CTD might also function to remove DNA-binding proteins during elongation, to facilitate post-transcriptional RNA processing, or to localize the transcriptional machinery to the nucleus (Corden, 1992).

RNA pol II cannot accurately initiate transcription by itself, but requires other associated proteins, termed transcription factors (TFs). The steps involved in assembly of transcription factors at a promoter are illustrated in Figure 1. This assembly of RNA pol II and its associated factors into a transcription complex at a promoter is a highly ordered process (Buratowski et al., 1989). The initial step is the binding of the TATA-binding protein (TBP) to the TATA box sequence, located approximately 30 basepairs upstream from

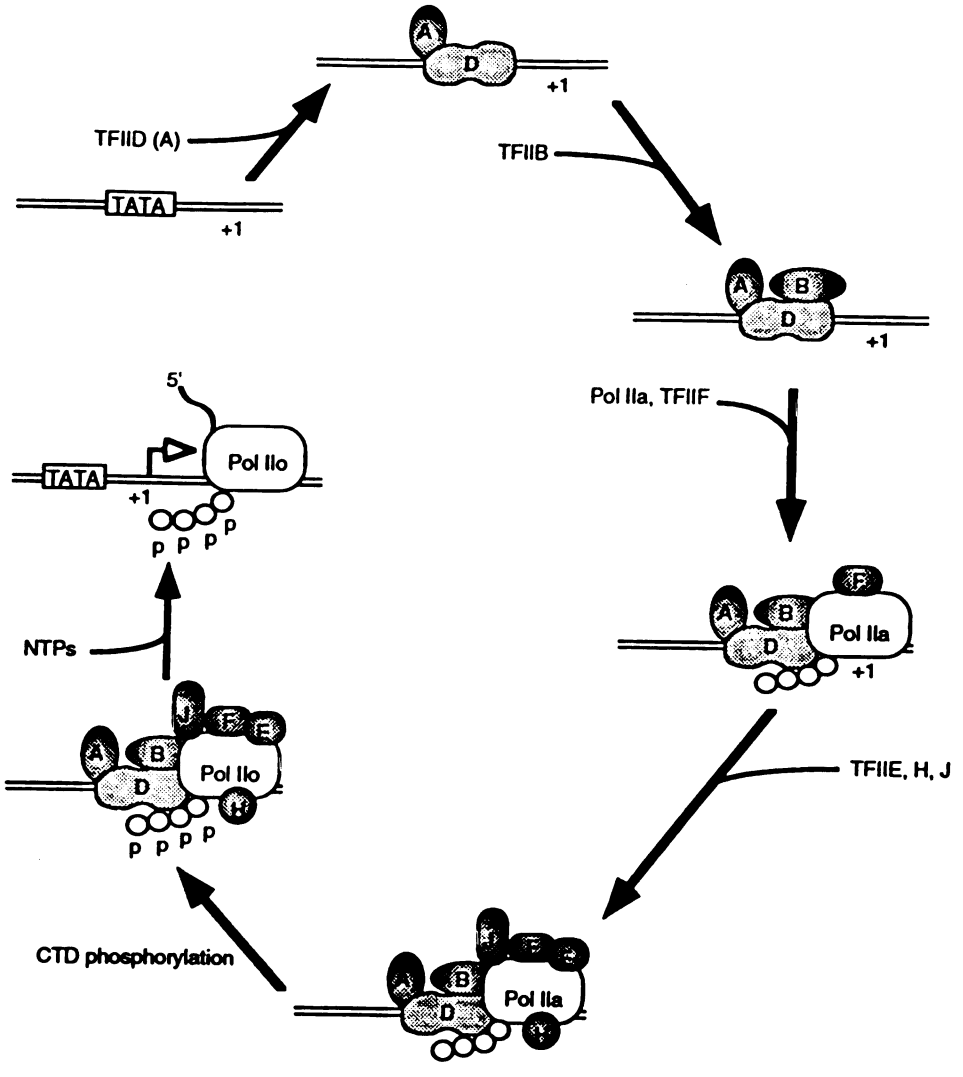

Figure 1. Assembly of basal transcription factors. The letters for the various transcription factors refer to the order of elution from phosphocellulose chromatography columns.

the transcription startsite (Davison et al., 1983; Fire et al., 1984; Reinberg et al., 1987). The gene for the TBP has been cloned from a number of different organisms, including human (Hoffmann et al., 1990; Kao et al., 1990; Peterson et al., 1990), yeast (Cavallini et al., 1989; Eisenmann et al., 1989; Hahn et al., 1989; Schmidt et al., 1989), Drosophila (Hoey et al., 1990), and Arabidopsis (Gasch et al., 1990). Sequence comparisons between the different TBPs revealed that the carboxyl-terminal half of TBP is highly conserved among species; for example, the carboxyl-terminal 180 amino acids of human and Drosophila TBPs are 88% identical (Zawel and Reinberg, 1992). In contrast, the amino-terminal region of TBP is greatly different between species, both in length and in amino acid sequence.

In higher eukaryotes, TBP does not bind to the TATA sequence as a monomer but rather as a large multisubunit protein complex denoted TFIID (Reinberg et al., 1987). Endogenous Drosophila TBP, for example, exists as a complex with at least six tightly associated proteins, or TAFs (TBP associated factors) (Dynlacht et al., 1991). In addition to the TAFs, other factors can associate with TBP to inhibit transcription by RNA pol II (Meisterernst and Roeder, 1991; Meisterernst et al., 1991; Inostroza et al., 1992).

Transcription factor IIA has been the subject of much controversy and conflicting data. Depending upon the cell type (or laboratory) from which it was isolated, a chromatographic fraction containing TFIIA activity was or was not required for basal transcription, and the activity could be comprised of one, two, or three polypeptides (Zawel and Reinberg, 1992). Recent reports indicate that S. cerevisiae TFIIA is composed of two subunits (Ranish et al., 1992) and that TFIIA interacts with TFIID (Buratowski and Zhou, 1992; Lee et al., 1992).

A report from the Reinberg laboratory (Cortes et al., 1992) may finally

have shed light on the role of TFIIA. Using yeast TFIID affinity chromatography, they purified TFIIA from HeLa cells and showed that it consists of three subunits, two of which correspond to those from TFIIA of S. cerevisiae. TFIIA stimulates basal transcription when a native HeLa TFIID is used, but not when transcription is reconstituted using bacterially produced TBP (Cortes et al., 1992). Thus they hypothesize that TFIIA counteracts the effects of a negative component which is normally associated with TFIID in vivo.

Transcription factor IIB, like TFIID, is absolutely required for basal transcription (Reinberg and Roeder, 1987a). TFIIB consists of a single polypeptide of approximately 30 kDa (Reinberg and Roeder, 1987a), the gene for which has been cloned from human, yeast, and Drosophila (Ha et al., 1991; Pinto et al., 1992; Wampler and Kadonaga, 1992; Yamashita et al., 1992). The TFIIB protein sequence shows a motif similar to one in prokaryotic sigma factors (Ha et al., 1991). This protein binds to the TFIID-DNA complex (Maldonado et al., 1990), but can also bind to RNA pol II. TFIIB is capable of suppressing nonspecific transcription by RNA pol II, probably by a direct interaction between TFIIB and RNA pol II (Wampler and Kadonaga, 1992). Although required for transcription, excess TFIIB can inhibit transcription from some promoters in vitro, perhaps by sequestering an unidentified basal transcription factor required by the repressed promoters (Wampler and Kadonaga, 1992). It has also been suggested that a possible function of transcriptional activator proteins (discussed in detail below) may be to induce a conformational change in TFIIB that allows it to interact more stably with RNA pol II (Colgan et al., 1993).

Transcription factor IIF (also known as RAP30/74) consists of two polypeptides of 30 kDa and 74 kDa (Flores et al., 1988). This factor binds tightly

to RNA pol II in solution (Reinberg and Roeder, 1987a), and it recruits RNA pol II to the TFIID-B-DNA complex. The small subunit of TFIIF is sufficient to recruit RNA pol II to the preinitiation complex (Flores et al., 1991) and it prevents RNA pol II from binding nonspecifically to DNA (Killeen and Greenblatt, 1992). The large subunit of TFIIF possesses amino acid sequence similarity to the E. coli transcription factor sigma 70 (McCracken and Greenblatt, 1991); also, this subunit is not required for transcription initiation, but it is required for elongation of mRNA transcripts (Chang et al., 1993).

Transcription factor IIE consists of two subunits of 57 kDa and 34 kDa in humans (Inostroza et al., 1991). TFIIE enters the preinitiation complex after the binding of RNA pol II and TFIIF (Inostroza et al., 1991). The large subunit contains a zinc finger motif and a domain similar to the catalytic loop of protein kinase C (Ohkuma et al., 1991; Peterson et al., 1991). The small subunit contains a putative nucleotide binding site (Peterson et al., 1991). Thus TFIIE is a potential CTD kinase. However, homogeneous preparations of TFIIE exhibited no ATPase activity, prompting the notion that other transcription factors are required for TFIIE function (Inostroza et al., 1991). It has also been reported that TFIIE increases the processivity of another potential CTD kinase, TFIIH (Lu et al., 1992).

Transcription factor IIH is a multisubunit factor that appears to have two potential roles in transcription. Lu et al. (1992) reported that TFIIH possesses a kinase activity which specifically phosphorylates the CTD of RNA pol II in vitro. A second function proposed for TFIIH is that of a helicase, which separates the strands of a DNA duplex, an operation required for transcription as well as DNA replication. It has been reported that the 89 kDa subunit of human TFIIH possesses an ATP-dependent helicase activity (Schaeffer et al., 1993). This subunit was found to be identical to the product of the ERCC-3

gene, a gene encoding a protein involved in DNA excision repair which is mutated in patients suffering from xeroderma pigmentosa and Cockayne's syndrome (Buratowski, 1993; Schaeffer et al., 1993); this result is intriguing, since both DNA repair and transcription require DNA strand separation.

Each transcription factor mentioned thus far frequently requires the presence of the others for its function. It is also apparent that not all of the aforementioned factors are required for transcription at every pol II promoter; for example, TFIIE was not required for transcription from the immunoglobulin heavy chain (IgH) gene promoter in vitro (Parvin et al., 1992). Futhermore, the topological state of the DNA template can affect the transcription factor requirements. The IgH gene promoter can support transcription with only TBP, TFIIB, and RNA pol II if it is in a negatively supercoiled state (Parvin and Sharp, 1993). This requirement for a minimal number of transcription factors is presumably due to the free energy stored in a negatively supercoiled template, energy which can function to partially unwind the DNA and facilitate the transition from a "closed" to an "open" transcription complex (Parvin and Sharp, 1993). Further work is necessary before the generality of this phenomenon is known.

The transcription complex described above has been termed the basal transcription complex. This complex forms at the promoters of most genes transcribed by RNA pol II, and facilitates basal transcription. However, many promoters contain upstream sequences which allow the adjacent genes to be expressed at levels higher than basal transcription. Conversely, regulatory sequences known as silencers function to inhibit expression of their cognate genes. Different combinations of the upstream regulatory sequences, both transcriptional activators and silencers, at different promoters allow finely tuned regulation of transcription initiation (Renkawitz, 1990). Sequence

elements that positively regulate adjacent promoters are termed enhancers (or UASs in yeast) and are bound by a class of proteins known collectively as transcriptional activators (Ptashne, 1988; Mitchell and Tjian, 1989). Activated transcription by a particular activator protein is specific for those promoters containing the binding site (enhancer element) for that activator protein.

Transcriptional Activator Proteins

Transcriptional activator proteins have been identified in a variety of eukaryotic species, including Drosophila, yeast, mouse, and human. Many transcriptional activator proteins have two domains: one conferring specific association with promoter sequences, usually a DNA binding domain, and a second domain for regulatory function (Brent and Ptashne, 1985; Ptashne, 1988; Mitchell and Tjian, 1989). The DNA specificity domains of transcriptional activators generally fall into different motifs, defined by primary sequence or three-dimensional structure. Examples of these motifs include zinc fingers, homeodomains, helix-turn-helix, helix-loop-helix, and leucine zippers (Mitchell and Tjian, 1989).

Activator proteins have been grouped into several classes, based on the amino acid content of their activation domains. The first activator proteins identified possessed activation domains containing a high percentage of negatively charged residues, hence the name acidic activators (Mitchell and Tjian, 1989). Some examples of acidic activator proteins are GAL4 (Ma and Ptashne, 1987a), GCN4 (Hope and Struhl, 1986), and HAP4 (Forsburg and Guarente, 1989) from yeast, and VP16 from herpes simplex virus type 1 (HSV-1). The GAL4 protein potently stimulates the transcription of the galactose-inducible genes in yeast (Bram and Kornberg, 1985; Giniger et al., 1985; Johnston, 1987), while the GCN4 protein is involved in the coordinated

induction of 30-50 genes for amino acid biosynthesis (Hope and Struhl, 1986). Other transcriptional activators identified contain activation domains with high proline or glutamine content, such as CTF and Sp1, respectively (Courey and Tjian, 1988; Mermod et al., 1989). It must be emphasized that this classification system is based solely on primary amino acid sequence; a better system would group activator proteins based upon the mechanism of increasing transcriptional initiation.

Mechanisms of Transcriptional Activator Function

Several models have been proposed to explain how activators work. They may function by directly or indirectly contacting a component of the basal transcription complex, thereby speeding up the ordered assembly of that complex at the promoter (Ptashne, 1988; Buratowski et al., 1989; Lewin, 1990; Ptashne and Gann, 1990; Wang et al., 1992). Potential targets of activator proteins include many of the basal transcription factors, such as TFIID, TFIIB, TFIIH, and RNA pol II (Stringer et al., 1990; Ingles et al., 1991; Lin and Green, 1991; Lin et al., 1991).

Activators could also increase the rate of transcriptional initiation by relieving the inhibition caused by histones, thus allowing the transcription complex to form (Croston et al., 1991; Laybourn and Kadonaga, 1991; Workman et al., 1991; Felsenfeld, 1992; Kornberg and Lorch, 1992). With regard to histones, transcriptional activator proteins can increase transcription in two ways: by removing histones from the promoter (antirepression) and/or by facilitating assembly of a transcription complex (true activation) (Laybourn and Kadonaga, 1992). Activators can produce their antirepressive effects either by competing with histones for binding at a

promoter or by physically displacing histones from the promoter (Felsenfeld, 1992).

A third model posits that activators speed up the transition from a preinitiation or initiation complex into an elongation complex, allowing transcription to proceed (Rougvie and Lis, 1990; Spencer and Groudine, 1990; Kerppola and Kane, 1991). A specific example of this would be facilitating the phosphorylation of the CTD of the largest subunit of RNA pol II.

These models need not be mutually exclusive; in fact, evidence exists for the participation of the transcriptional activation domain of VP16 (a prototypical activator) in several of these mechanisms. Because an understanding of how activators function is crucial to our understanding of transcriptional regulation, we have undertaken a detailed and systematic mutagenesis study of VP16.

VP16

The expression of the genes of herpes simplex virus type 1 (HSV-1) occurs in a temporally regulated manner (Honess and Roizman, 1974; Honess and Roizman, 1975). Once inside the host cell, the HSV-1 virion is transported to the nucleus, at which point the viral DNA is released into the nucleus. During the lytic cycle, the first HSV-1 genes to be expressed are the immediate early (IE) genes, followed by the early genes and then the late genes (Honess and Roizman, 1974). The five immediate early genes encode transcriptional regulatory factors, and the expression of these genes is enhanced by the virus-encoded transcriptional activator protein VP16 (also known as α-TIF and Vmw65) (Post et al., 1981; Campbell et al., 1984). VP16 consists of 490 amino acids and has an apparent molecular weight of 65 kDa on SDS-PAGE. VP16 possesses two functional domains pertinent to its role in

transcription. The amino terminal region of the protein (the specificity domain) interacts with host cell factors that bind to IE gene promoter elements (McKnight et al., 1987; Gerster and Roeder, 1988; O'Hare and Goding, 1988; O'Hare et al., 1988; Preston et al., 1988; Triezenberg et al., 1988a). One of the host factors is Oct-1, a protein which binds to the octamer consensus sequence ATGCAAAT and is found in all tissue types; the octamer consensus sequence frequently overlaps the consensus DNA-binding sequence (TAATGARAT) for VP16 in HSV-1 IE promoters (Sturm et al., 1988; Stern et al., 1989). A second host factor, variously called C1, VCAF-1, CFF, and HCF, is also required for VP16 to bind to the IE gene promoters (Kristie et al., 1989; Katan et al., 1990; Xiao and Capone, 1990). The second functional domain of VP16 is involved in transcription activation and resides in the carboxyl terminal 80 amino acids of the protein, and is both necessary and sufficient for activating transcription (Sadowski et al., 1988; Triezenberg et al., 1988b; Cousens et al., 1989; Greaves and O'Hare, 1989; Werstuck and Capone, 1989). The transcriptional activation domain of VP16 contains a high percentage of acidic residues, and has a net negative charge of -21 (Figure 2).

Models for Transcriptional Activation by VP16

Much of the research in our lab to date has been focused on elucidating those structural elements critical for the function of the VP16 transcriptional activation domain. The groundwork for my research was laid by Doug Cress (Cress and Triezenberg, 1991a), and will be briefly described below.

Two theories have been proposed to explain key structural elements of acidic activation domains such as that of VP16. The first theory, proposed by crystallographer Paul Sigler (1988), was based on the apparent lack of sequence similarity among acidic activation domains, and suggested that the strength

L S T A P P T **D** V S L G **D** **E**
CTG TCG ACG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG
410 420

L H L **D** G **E** **D** V A M A H A **D** A
CTC CAC TTA GAC GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG
430

L **D** **D** F **D** L **D** M L G **D** G **D** S P
CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG
440 450

G P G F T P H **D** S A P Y G A L
GGT CCG GGA TTT ACC CCC CAC GAC TCC GCC CCC TAC GGC GCT CTG
I 460
Δ456

D M A **D** F **E** F **E** Q M F T **D** A L
GAT ATG GCC GAC TTC GAG TTT GAG CAG ATG TTT ACC GAT GCC CTT
470 480

G I **D** **E** Y G G END
GGA ATT GAC GAG TAC GGT GGG TAG
490

Figure 2. Nucleotide and deduced amino acid sequences of the VP16 activation domain (codons 410-490). Bold letters indicate acidic residues; outline letters indicate phenylalanine residues. The truncated VP16 activation domain (del456) is indicated.

of a given acidic activation domain resulted primarily from its net negative charge. No secondary structure was necessary, hence the term "negative noodle." The second theory, advocated by Mark Ptashne (Giniger and Ptashne, 1987), proposed that acidic activation domains form amphipathic alpha helices (AAHs), with acidic residues forming one face of the helix and hydrophobic residues lying along another face of the helix. To support their model, Giniger and Ptashne designed a 15 amino acid peptide which, if in the form of an alpha helix, would be both amphipathic and acidic. A gene fusion was made between sequences encoding the GAL4 DNA-binding domain (aa 1-147) and those encoding the 15 amino acid peptide. This fusion protein was capable of activating transcription from a test promoter. A peptide having the same residues but in scrambled order such that it would not form an AAH was not able to function as a transcriptional activation domain (Giniger and Ptashne, 1987). However, no evidence was provided that the 15 amino acid peptide actually formed an alpha helix.

Doug Cress set out to test these two disparate theories, using site-directed mutagenesis to target specific residues in the VP16 activation domain. A VP16 activation domain truncated after amino acid 456 (del456) was used as the mutagenesis substrate. To test Sigler's "negative noodle" theory, acidic amino acids in the truncated VP16 activation domain were removed and replaced with their neutral counterparts, in increasing number and in various combinations. In general, a decrease in net negative charge was accompanied by a decrease in activator function (Cress and Triezenberg, 1991a). However, some combinations of changes possessing identical charge had significantly different activities, which contradicts the Sigler theory that net charge is the primary determinant of activator strength. This result suggested, perhaps not surprisingly, that some element of structure was an

important contributor to VP16 activation function.

The Ptashne model, which suggested a specific secondary structure for acidic activation domains, was then tested. According to standard secondary structure prediction algorithms (Chou and Fasman, 1978; Garnier et al., 1978), the VP16 activation domain has the potential to form an alpha helix, and such a helix would be amphipathic. Groups of four acidic residues were replaced with neutral counterparts in a circularly permuted manner around the putative helix, generating what were termed "face" mutants. The assumption was that removal of negative charge from the center of the acidic face of an amphipathic alpha helix would be more detrimental to activity than removal of negative charge from the edges of the acidic face. However, no correlation was observed between predicted amphipathy and transcriptional activity of VP16 (Cress and Triezenberg, 1991a). For example, several mutants in which charge was removed from the center of the acidic face had more activity than mutants in which charge was removed from the edges of the acidic face.

Since the amphipathy of the VP16 activation domain did not appear to contribute to its activity, additional mutations were made to determine if alpha helical structure was necessary for function. The amino acid proline is generally incompatible with alpha helical structure because the proline side chain is bonded to the nitrogen atom of the polypeptide backbone, which prevents the participation of the nitrogen in hydrogen bonding necessary to stabilize the helix (Creighton, 1984). Insertion of a potentially helix-breaking proline residue at position 425 of the truncated VP16 activation domain had no effect on activity; furthermore, substitution of two prolines simultaneously at positions 432 and 436 within the predicted alpha helix also had no effect on activity (Cress and Triezenberg, 1991a). Conversely, replacing

a phenylalanine at position 442 with proline abolished activity. Replacing Pro442 with helix-compatible but non-aromatic amino acids (Ala, Ser) did not restore activity to the VP16 activation domain. However, substitution of an aromatic amino acid, tyrosine, at position 442 restored activity to approximately 30% of VP16 del456 activity (Cress and Triezenberg, 1991a).

After the discovery of the importance of Phe442, the amino acid sequence of the VP16 activation domain was compared to the sequences of other transcriptional activation domains. The sequences of several transcriptional activation domains were aligned using as a guide six bulky hydrophobic residues of the VP16 activation domain (Cress and Triezenberg, 1991a; Figure 3). In a number of different types of activation domains, bulky hydrophobic residues are observed at positions that can be aligned with Leu439 and Leu444 of VP16, on either side of the critical Phe442 (Figure 3).

Overview

Cress and Triezenberg (1991a) demonstrated the importance of Phe442 for the activity of the VP16 protein truncated after amino acid 456. Their results also suggested that the important characteristic of phenylalanine at this position was its aromaticity. In the following chapter, I describe work using site-directed mutagenesis to further our understanding of the critical Phe442 and of other regions of the VP16 activation domain important to its ability to activate transcription. The phenylalanine at position 442 of the truncated (del456) VP16 activation domain was thoroughly mutagenized to obtain nearly all other amino acid substitutions at this position. The results of this mutagenesis of position 442 confirm the hypothesis of Cress and Triezenberg (1991a) that the aromatic character at this position is critical; truncated VP16 proteins containing either of the other two aromatic amino

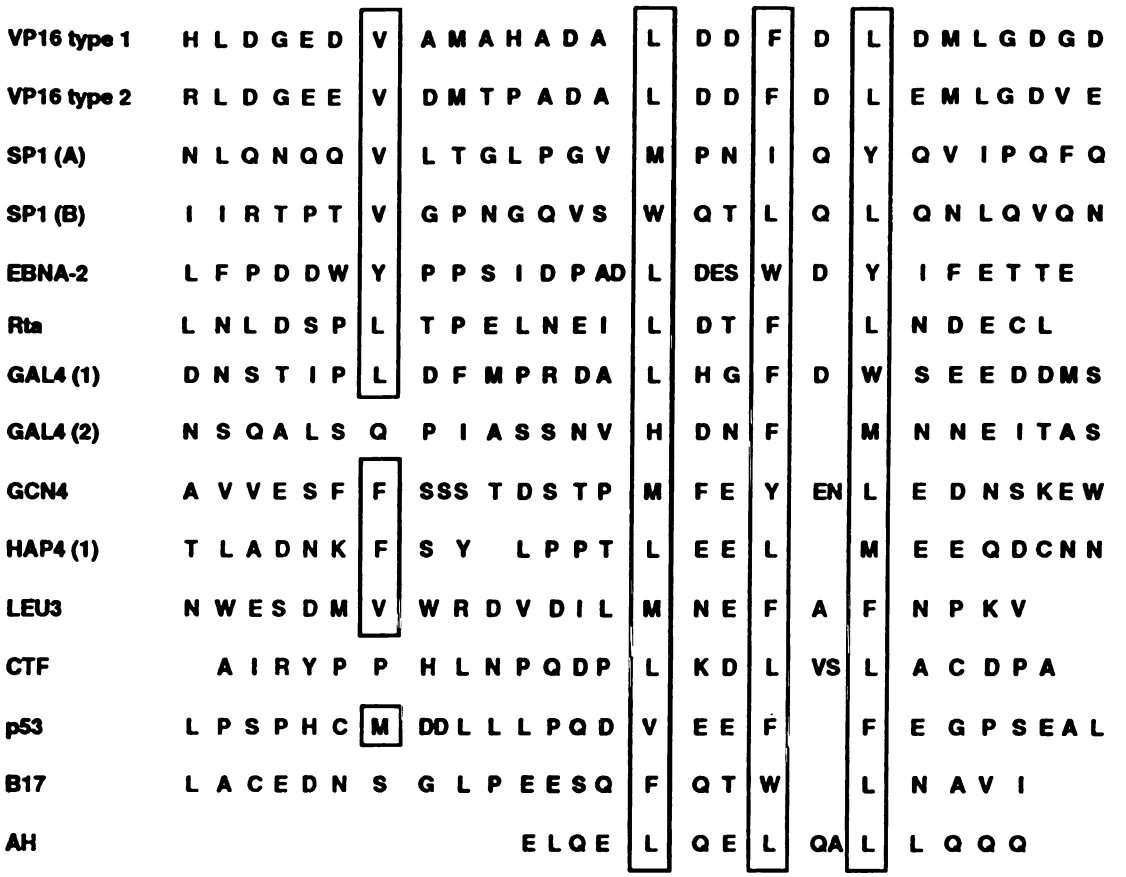

Figure 3. Alignment of hydrophobic residues from various transcriptional activation domains with that of VP16 (revised and extended from Cress and Triezenberg, 1991a). VP16 type 1 and 2 refer to VP16 proteins from HSV-1 and HSV-2 (Cress and Triezenberg, 1991b), respectively. Sp1 (Courey and Tjian, 1988) and CTF (Mermod et al., 1989) are mammalian glutamine- and proline-rich transcriptional activators, respectively. EBNA-2 and Rta are activators from Epstein-Barr virus (Cohen, 1992; Hardwick et al., 1992). GAL4 (Ma and Ptashne, 1987a), GCN4 (Hope and Struhl, 1986), HAP4 (Forsburg and Guarente, 1989), and LEU3 (Zhou et al., 1987) are yeast activators. p53 is a human tumor suppressor protein (Harlow et al., 1985). B17 is a randomly-cloned E. coli genomic fragment (Ma and Ptashne, 1987b). AH is an artificial sequence designed to form an amphipathic alpha helix (Giniger and Ptashne, 1987).

acids (Tyr and Trp) were approximately 30-35% active relative to wild type del456. Hydrophobic residues supported an activity level 10-15% of wild type del456, while all other amino acids tested at this position (with the exception of asparagine) were less than 10% active.

The pattern of bulky hydrophobic residues observed by Cress and Triezenberg suggested that leucines at positions 439 and 444 of VP16 may be important for transcriptional activity (Figure 3). These two leucine residues of VP16 were also targeted for mutagenesis; substitution of small hydrophobic or hydrophilic amino acids for either leucine greatly decreased activity, a result which is supportive of the Cress model.

We also show that the addition of amino acids 457-490 to the truncated VP16 activation domain partially restores the activities of truncated VP16 mutants inactivated by changes at Phe442, implying functional redundancy within the full-length VP16 activation domain. Within this added region, a phenylalanine at position 473 is found in a context similar to that surrounding Phe442. Changing this residue to non-aromatic amino acids only modestly affected the activity of this region. Instead, mutations at a neighboring phenylalanine (at position 475) had somewhat greater effects on activity, despite it not agreeing as well as Phe473 with the Cress model. Our work suggests that two regions of the VP16 activation domain have different structural elements and may have different mechanisms for achieving transcriptional activation.

Our lack of success in identifying amino acids important to the function of the distal subdomain of the VP16 activation domain led us to try a more efficient method for generating mutations in VP16 and testing their effects on the ability of VP16 to activate transcription. In Chapter III I describe a method of randomly mutating the VP16 activation domain using DNA-

damaging chemicals, which, combined with an in vivo selection in yeast, enabled us to identify mutations in residues of the VP16 activation domain which decrease function. This strategy identified the region of VP16 between amino acids 480 and 485 which is important for the function of the distal subdomain.

In Chapter IV I discuss our current model of how the two subdomains of the VP16 activation domain might function to activate transcription. This model is based upon our mutagenesis results and the results of collaborations in which the various VP16 mutants described here were used to investigate specific aspects of activated transcription.

CHAPTER II

SITE-DIRECTED MUTAGENESIS OF THE VP16 TRANSCRIPTIONAL ACTIVATION DOMAIN

Introduction

Previous work by Cress and Triezenberg (1991a) revealed the importance of Phe442 to the activity of the truncated VP16 activation domain (del456), and also suggested that bulky hydrophobic residues surrounding Phe442 (Leu439 and Leu444) might also contribute to the activity of VP16 del456. Using site-directed mutagenesis, I extended this work by generating and testing further mutations at Phe442; the results of further Phe442 substitutions suggests that an aromatic or hydrophobic residue is critical at this position. Substitutions of small hydrophobic or hydrophilic amino acids for Leu439 or Leu444 greatly reduced activity of VP16 del456. I also tested the activities of a variety of mutations at Phe442 in the context of the full-length VP16 activation domain. The full-length Phe442 mutants had increased activity compared with their truncated counterparts, suggesting that the added carboxyl-terminal region (aa 457-490, designated the distal subdomain) contributes to the overall activity of the VP16 activation domain. A pattern of amino acids similar to that surrounding Phe442 was observed in the added region. I tested the hypothesis that Phe473 (or possibly Phe475) was analogous to Phe442; however, mutations at these phenylalanines in the distal subdomain did not decrease activity to the same degree as similar mutations at Phe442, suggesting that the proximal and distal subdomains activate

transcription through different mechanisms. These results were published in the Proceedings of the National Academy of Sciences USA (1993, Vol. 90, pp. 883-887).

Chapter II Methods

Mutagenesis and Cloning

SalI/BamHI fragments of the VP16 gene corresponding to the truncated (codons 411-456) or full-length (codons 411-490 plus 7 bp of 3' nontranslated sequence) activation domain were cloned into M13mp19 (Norrander et al., 1983). Oligonucleotide-directed mutagenesis was performed as described (Zoller and Smith, 1982; Kunkel, 1985; Cress and Triezenberg, 1991a). I prepared single-stranded uridine-containing template DNA by replicating the template in the E. coli host strain CJ236 [dut-1, ung-1, thi-1, relA-1; pCJ105(Cm^r F')] which results in occasional uridines incorporated in place of thymidines in all DNA synthesized in the bacterium (Bio-Rad Muta-Gene in vitro mutagenesis kit instruction manual, catalog #170-3571). The uracil-containing single-stranded DNA was purified by extensive phenol/chloroform and chloroform extraction, Biogel P-60 column chromatography, and ethanol precipitation. The uracil-containing single-stranded DNA was resuspended in 20 µl of TE (10 mM Tris-Cl, pH 7.5, 1 mM EDTA).

Second-strand synthesis was performed in vitro using a phosphorylated mutagenic oligonucleotide as a primer (Table 1). In a reaction volume of 10 µl, 1 µl (approximately 6 pmole) of phosphorylated primer was combined with 1 µl of uridine-containing template DNA in annealing buffer (20 mM Tris-Cl, pH 7.5, 2 mM $MgCl_2$, and 50 mM NaCl) and the annealing accomplished by heating the sample to 65°C and cooling gradually to 4°C.

I.D.	Sequence	Mutation
ST-17	5'-GTCCAGATCC(a/c)AATCGTCTAA-3'	F442 to L, W
ST-18	5'-GAAATCGTCA(a/g)(a/c)CGCGTCGGC-3'	L439 to F, S, V, A
ST-19	5'-CAACATGTC(t/a)(a/g)(c/a)ATCGAAATC-3'	L444 to F, S, V, A
ST-21A	5'-CTCAAACTCA(g/a)(g/c)GTCGGCCAT-3'	F473 to P, A, V, L
ST-22	5'-CATCTGCTCA(g/a)(g/c)CTCGAAGTC-3'	F475 to P, A, V, L
ST-25	5'-GTCCAGATC(c/a)(t/a/c)(t/g/c)ATCGTCTAA-3'	F442 to K, N, M, I, R, Q, H, E, D, V, G, L, S
ST-26	5'-CATCTGCTCA(g/a)CCTCGAAGTC-3'	F475 to V, A
ST-27	5'-CTCAAACTC(c/a)(c/t)AGTCGGCCAT-3'	F473 to W, Y, C
ST-28	5'-CAACATGTCCACATCGAAATC-3'	L444 to V
ST-29	5'-CATCTGCTC(g/c)(t/c)ACTCGAAGTC-3'	F475 to W, Y, C

Table 1. Mutagenic oligonucleotides. The sequences of the oligonucleotides used to alter the VP16 gene are listed in this table. Parentheses indicate mixed positions. The specific mutations conferred by each oligonucleotide are also indicated. Fan Shen performed the mutagenesis with ST-25. These oligonucleotides were synthesized by MSU Department of Biochemistry Macromolecular Structure Facility and used without further purification.

After annealing of the primer, the complementary DNA strand synthesis was performed by adding 1 µl of 10X extension buffer (175 mM Tris-Cl, 37.5 mM $MgCl_2$, 215 mM DTT, 7.5 mM ATP, 4 mM each deoxynucleotide triphosphate) and 1 µl (1 unit) each of T4 DNA polymerase and T4 DNA ligase. The reactions were incubated on ice for 5 minutes, then at 25°C for 5 minutes, and finally at 37°C for 1.5 hours; the reactions were terminated by addition of 87 µl of TE.

Mutagenic synthesis reactions were then used to transform dut^+ ung^+ MV1193 cells [Δ(lac-pro AB), rpsL, thi, endA, spcB15, hsdR4, Δ(srl-rec A)306::Tn10(tet^r) F'(traD36, $proAB^+$, $lac I^q$ lacZΔM15)], made competent by treatment with 100 mM $CaCl_2$. Transformation of the mutagenized duplex DNA into MV1193 results in selection against the uracil-containing DNA strand due to the action of uracil N-glycosylase. The resulting plaques were purified by picking one with a small pipette tip. Each tip was used to inoculate a 3 ml culture of a 1:60 dilution of an overnight culture of MV1193 in LB media.

Mutations were identified by dideoxy sequencing (Sanger et al., 1977) and double-stranded phage DNAs containing the desired mutant VP16 activation domains were harvested. The SalI/BamHI fragments of VP16 encoding the altered activation domains were cloned downstream of codons 1-410 using the expression vector pMSVP16 (Triezenberg et al., 1988b). After cloning into the expression vector each mutant VP16 activation domain was sequenced to ensure fidelity.

Transient transfection assay

Mouse L cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum (HyClone Laboratories). Cells ($8x10^5$ per 60-mm culture plate) were transfected by

DEAE-dextran (Lopata et al., 1984). In this method, DEAE-dextran at 200 μg/ml was dissolved in DMEM supplemented with 10 mM HEPES, pH 7.2. This solution was combined with the following plasmids: an expression plasmid pMSVP16 (50 ng), a reporter plasmid (2 μg of pSJT 703) containing the ICP4 IE regulatory sequences fused to the body of the HSV-1 tk gene, and an internal control plasmid (2 μg of pMSV-tk) containing the MSV-LTR promoter fused to the tk gene body (Graves et al., 1985). The reporter and internal control tk genes differed in the location of the transcription startsite. Cells were incubated with the DEAE-dextran/DNA solutions for 5 hours at 37°C.

At the end of the incubation period the cells were shocked with a dimethyl sulfoxide solution (10% DMSO, 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 0.1% dextrose, 20 mM HEPES) for 3 minutes. After the DMSO shock, the cells were washed with 2 ml DMEM/HEPES, after which 5 ml of DMEM supplemented with 10% fetal calf serum was added and the plates incubated at 37°C. Total RNA was harvested 48 hours after transfection as described (Triezenberg et al., 1988b).

For the RNA harvest, solutions were made RNAase-free whenever possible. Transfected cells were incubated with 2.5 ml of a 200 μg/ml proteinase K solution (10 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1% SDS), the cells were scraped off the plates into 15 ml culture tubes, and the samples incubated at 50°C for 3 hours. The samples were extracted two times with phenol (saturated with DEPC-treated water) followed by chloroform. Nucleic acids were precipitated by addition of ethanol followed by centrifugation. The pellets were resuspended in 400 μl of TE and transferred to 1.5 ml microfuge tubes. The ethanol precipitation was repeated, and the final nucleic acid pellets resuspended in 100 μl TE. The samples were digested for 2 hours at

37°C with DNAase I (5 µg DNAase I, 50 µg heparin, 20 mM HEPES, pH 7.2, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $MnCl_2$). Following the DNAase I digestion, the samples were extracted again with phenol and chloroform and ethanol precipitated. RNA pellets were washed with 70% ethanol and resuspended in 25 µl of TE.

Primer extension assay

The tk primer used in the primer extension assay was phosphorylated by combining 45 ng of tk oligonucleotide, 30 µCi of [γ-^{32}P]-ATP, and 15 units of T4 polynucleotide kinase in kinase buffer (70 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$), in a reaction volume of 10 µl. The kinase reaction was incubated at 37°C for 2 hours, and the reaction stopped by addition of 2 µl of 0.5 M EDTA and 50 µl of TE, followed by heating at 65°C for 5 minutes. Unincorporated ATP was removed from the labeled tk primer solution by binding the oligonucleotide to a DEAE-cellulose column in TEN100 buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 100 mM NaCl), washing with 1 ml of TEN100 and then 500 µl of TEN300 (TE plus 300 mM NaCl), and finally eluting the labeled oligonucleotide with 400 µl of TEN600 (TE plus 600 mM NaCl). The sample was frozen at -20°C until use.

Ten µl of each RNA sample was incubated with 3.5 µl of the ^{32}P-labeled tk primer in a buffer composed of 150 mM KCl, 10 mM Tris-Cl, pH 8.3, and 1 mM EDTA. The annealing was performed at 65°C for 1.5 hours, after which the samples were allowed to cool to 25°C. The primer extension reaction was performed by adding 30 µl of primer extension reaction buffer [5 units AMV reverse transcriptase (Life Sciences, Inc.), 20 mM Tris-Cl, pH 8.3, 10 mM $MgCl_2$, 6 mM DTT, 150 µg/ml actinomycin D] to the annealed primer solution, and incubation at 37°C for 1 hour. The samples were treated with 20 µg/ml RNAse A plus 100 µg/ml salmon sperm DNA for 15 minutes at 37°C,

and then extracted with phenol/chloroform and ethanol precipitated. Primer extension products were resuspended in 10 µl of formamide loading dye (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue and xylene cyanol). The ICP4-tk and MSV-tk RNAs yielded primer extension products of 81 and 55 bases, respectively; these products were separated by electrophoresis on a 9% acrylamide denaturing (7 M urea) gel, 0.5X TBE buffer, and detected by autoradiography.

Quantitation of primer extension products

The developed film and the dried gel were aligned and portions of the gel corresponding to bands on the film were cut out. Cerenkov activity of each gel slice was detected by a 5 minute scan on the tritium setting of a Packard 300 liquid scintillation counter. Alternatively, the dried gel was used to expose a phosphor screen, and the image detected by a Molecular Dynamics PhosphorImager. Activity of each band was determined using the ImageQuant program and volume integration with correction for background.

Determination of mutant protein stability

Mouse L cells ($8x10^5$ per 60-mm culture plate) were transfected with 10 µg of wild-type or mutant pMSVP16 as described above. Forty eight hours after transfection, the cells were lysed in 2 ml of SDS buffer (50 mM Tris, 0.2 M NaCl, 20 mM EDTA, 0.5% SDS) and scraped into 15 ml polystyrene conical tubes and sonicated. After sonication, the samples were transferred to 15 ml polypropylene conical tubes, and total protein was precipitated with 8 ml of cold acetone. The protein pellets were resuspended in 200 µl of SDS-PAGE sample buffer (62 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol); approximately 40 µl of each sample was electrophoresed on a 4% stacking/10% resolving SDS-PAGE gel. Proteins were electrophoretically

transferred to nitrocellulose using a Western Mini Transphor TE22 apparatus (Hoefer Scientific) and VP16 protein was detected by C8-series anti-VP16 antisera from rabbit (Triezenberg et al., 1988b). Primary antibody was visualized using a biotinylated goat anti-rabbit secondary antibody and avidin/biotinylated enzyme complex, the substrate for which was 4-chloro-1-naphthol (Vector Labs).

RESULTS

Mutagenesis of Phe442

Cress and Triezenberg (1991a) suggested that Phe442 is critical for function of the truncated VP16 activation domain (del456). To more thoroughly test this hypothesis, additional amino acid substitutions were made at position 442. Fan Shen in our laboratory was responsible for the generation and testing of eleven Phe442 mutants; I made and tested the FW442 and FL442 mutants, while the remaining mutants have been reported previously (Cress and Triezenberg, 1991a). Activities of VP16 mutants were determined by transient transfection assays in which a plasmid expressing the VP16 gene was cotransfected with a reporter plasmid bearing the HSV-thymidine kinase (tk) gene under the control of a VP16-responsive promoter and an internal control plasmid consisting of the tk gene regulated by the MSV LTR promoter. Total RNA was harvested and the amount of HSV-tk RNA was quantitated by primer extension assay and scintillation spectroscopy. Of the nineteen possible changes at position 442, seventeen have been generated and their activities tested. Substitution of the other two aromatic amino acids, tyrosine and tryptophan, for Phe442 decreased but did not abolish function (Figure 4). No other substitution mutants had an activity greater than fifteen percent relative to wild type del456 (Table 2). The

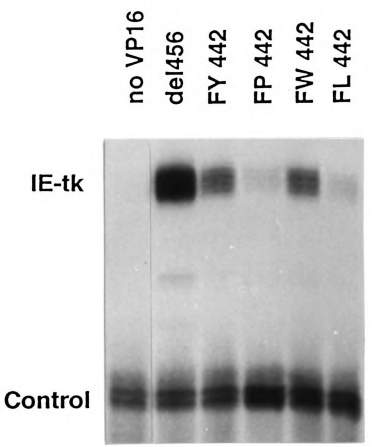

Figure 4. Autoradiogram of primer extension assay reflecting the activities of truncated VP16 proteins altered at Phe442. Positions of the reporter (IE-tk) and internal control primer extension products are indicated. The size and stability of all mutant proteins were confirmed by Western blotting and immunodetection by anti-VP16 antisera (see Figure 5).

Amino acid at Position 442	Relative activity (% of wt del456)
Tyr*	30 ± 10
Trp	36 ± 6
Leu	14 ± 2
Pro*	≤10
Ala*	14 ± 3
Ser*	≤10
Gly	≤10
Val	11 ± 2
Ile	14 ± 2
Met	12 ± 1
Lys	≤10
Arg	≤10
His	≤10
Asp	≤10
Glu	≤10
Asn	11 ± 2
Gln	≤10

Table 2. Relative activities of truncated (del456) VP16 mutants bearing amino acid substitutions at position 442. Relative activities are calculated as the ratio of the reporter signal (IE-tk) to the internal control signal, normalized to the activity of wild type del456. Means and standard deviations were calculated from at least four independent transfections. Asterisks indicate activities previously reported (Cress and Triezenberg, 1991a). Fan Shen is responsible for all the data in this figure except FW442, FL442, and those identified with an asterisk.

substitution of bulky hydrophobic residues (Leu, Ile, and Met) and the smaller hydrophobic residue Ala reduced activity to approximately ten to fifteen percent of del456 activity. All other substitution mutants were no more than ten percent active, with the exception of the asparagine substitution (11%). Interestingly, increasing the net negative charge by substitution of acidic residues at position 442 had no positive effect. To determine the stability of mutant proteins, immunoblots were probed with polyclonal antisera directed against VP16, an example of which is shown in Figure 5. All of the mutant proteins described in this dissertation showed no significant differences in size or stability from their wild type VP16 parent. These results strongly support the suggestion stated previously (Cress and Triezenberg, 1991a) that the aromatic character of amino acid 442 is particularly important, and that hydrophobic residues at this position are less effective but retain some function.

Hydrophobic Residues Flanking Phe442 Also Contribute to Activity

Alignment of the amino acid sequences of several transcriptional activation domains revealed an intriguing pattern of bulky hydrophobic residues (Cress and Triezenberg, 1991a). In the VP16 activation domain such residues include leucines at both positions 439 and 444 flanking Phe442 (Figure 3). To determine if these leucines contribute to the activity of the truncated VP16 activation domain, single amino acid substitutions were made and tested in transient transfection assays. Substitution at either position of a small hydrophobic (Ala) or a hydrophilic residue (Ser) for leucine diminished activity significantly (Figure 6). Substitution of another bulky hydrophobic residue (Val) or an aromatic hydrophobic residue (Phe) decreased activity only slightly. A bulky hydrophobic amino acid was perhaps

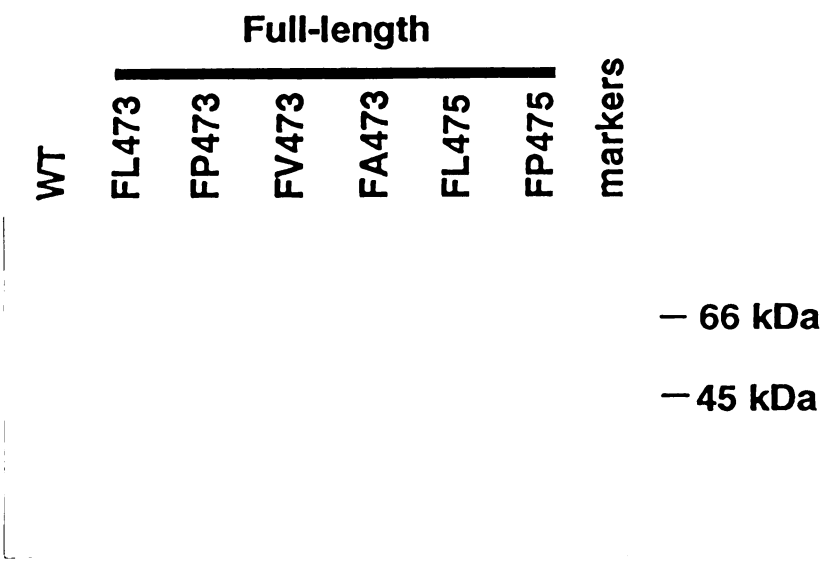

Figure 5. Determination of mutant protein stability. An example of wild type VP16 or mutant derivatives thereof harvested from transfected L cells, separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-VP16 antisera (see Methods for details). Protein standards sizes in kDa are indicated.

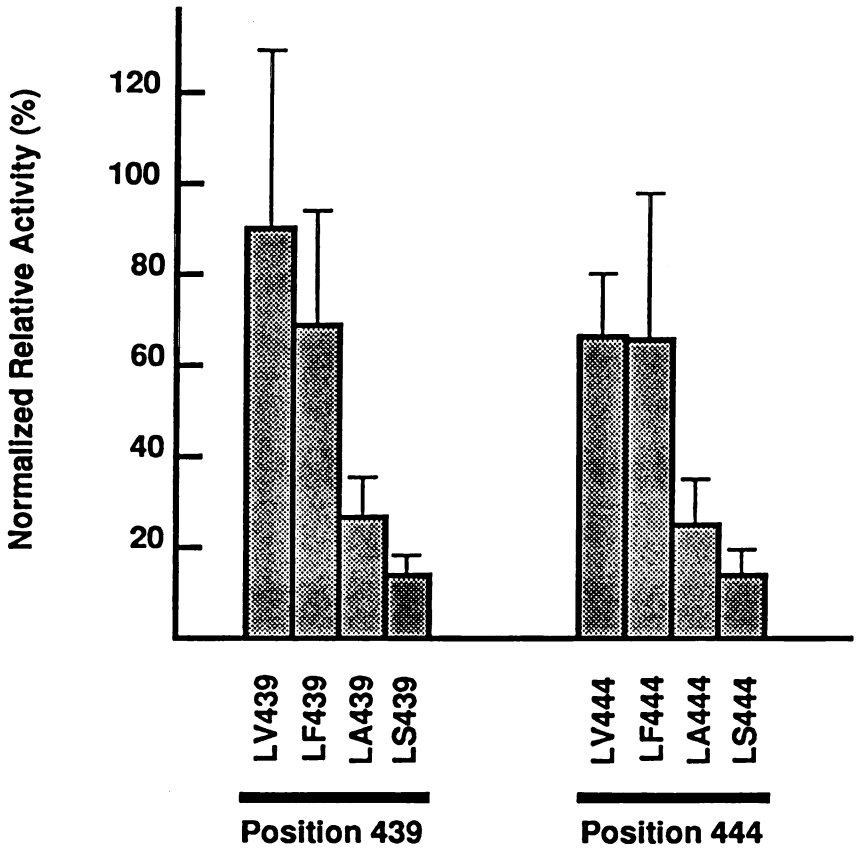

Figure 6. Effects of amino acid substitutions at Leu439 or Leu444 of truncated VP16. Rectangles indicate mean relative activities calculated (as for Table 2) from at least five independent transfections; error bars represent one standard deviation.

a slightly better substitute than an aromatic hydrophobic amino acid at either position. We conclude that the pattern of hydrophobic residues observed among different transcriptional activation domains is an indicator of amino acids important to the function of the truncated VP16 activation domain.

Addition of aa 457-490 Restores Partial Activity to Phe442 Mutants

All of the mutations described above were tested in a VP16 activation domain truncated after amino acid 456. Previous deletion analyses demonstrated that this truncated activation domain possesses approximately 50% of wild-type activity (Triezenberg et al., 1988b). We wished to know if the presence of the last 34 amino acids of the activation domain could restore activity to those truncated proteins inactivated by mutations at Phe442. Therefore we placed Phe442 substitutions in the context of the full-length activation domain. Addition of amino acids 457-490 partially restored activity to VP16 mutants such that relative activities of the full-length proteins reflected the activities of the corresponding truncated mutants (Figure 7). For example, truncated FA442 showed approximately 15% of the activity of its truncated parent, whereas full-length FA442 had activity approximately 45% of wild type full-length VP16. Similarly, truncated FW442, approximately 35% of del456 activity, was 70% of wild type VP16 upon addition of the remainder of the activation domain. Thus, replacing the distal domain restores partial activity to formerly defective del456 mutants.

Proximal and Distal Subdomains of VP16 Have Unique Structural Features

The results from adding back amino acids 457 to 490 suggested that the VP16 activation domain might possess some functional or structural

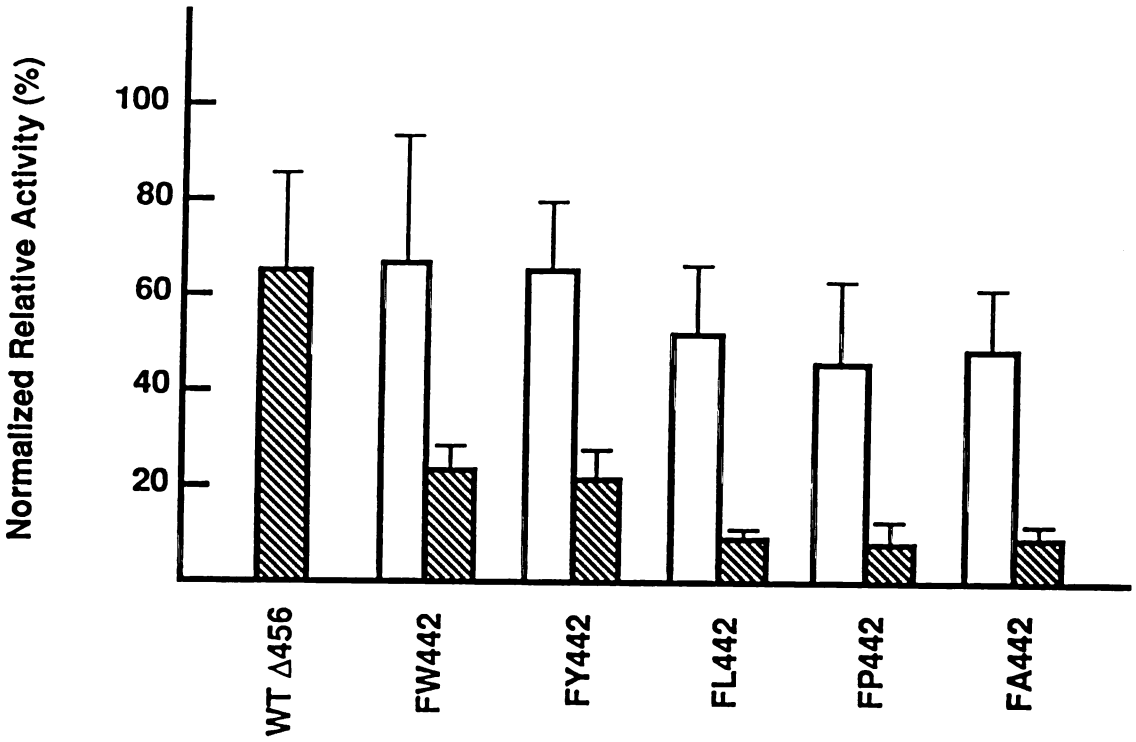

Figure 7. Relative activities of full-length and truncated VP16 mutants bearing substitutions at Phe442. Rectangles indicate mean relative activities calculated from at least five independent transfections; error bars represent one standard deviation. Activities for this figure have been normalized to the activity of full-length wild type VP16. Open rectangles represent full-length VP16 mutants; cross-hatched rectangles represent truncated mutants.

redundancy. Therefore, based on our knowledge of important amino acids surrounding Phe442, we examined the sequence of residues 457-490 and discerned a pattern of hydrophobic and aromatic residues similar to that surrounding Phe442 (Figure 8). This sequence similarity and the results of adding back aa 457-490 suggested that the activities of the proximal (aa 410-456) and distal (aa 457-490) subdomains of VP16 might rely on similar structural features. Two phenylalanines (Phe473 and Phe475) were identified within a pattern of acidic and hydrophobic residues similar to that surrounding Phe442. Our expectation, based on sequence similarity, was that Phe473 would be the counterpart of Phe442, and that mutations at Phe473 would therefore have a strong effect on the activity of the distal subdomain. Amino acid substitutions at Phe473 or Phe475 were made, and the activities of the mutants were tested in the context of the FA442 mutation or in the wild-type Phe442 background. We saw little or no effect of substitutions at positions 473 or 475 in the context of wild-type Phe442 (data not shown). Furthermore, when such substitutions were tested in the context of a FA442 mutation, we observed that the types of substitutions that inactivated the proximal subdomain had much less effect on the activity of the distal subdomain (Figure 9). Of the substitutions at position 473, the proline and alanine substitutions were not significantly less active than leucine or tyrosine substitutions.

Substitutions at position 475 had more pronounced effects. Proline or alanine substitutions decreased activity to approximately 50% of full-length FA442, while substitution of bulky hydrophobic residues leucine or valine decreased activity to approximately 80% and 65%, respectively. The substitution of a tyrosine residue had no significant effect on activity. Thus, similar kinds of mutations at corresponding positions in the 442 region and

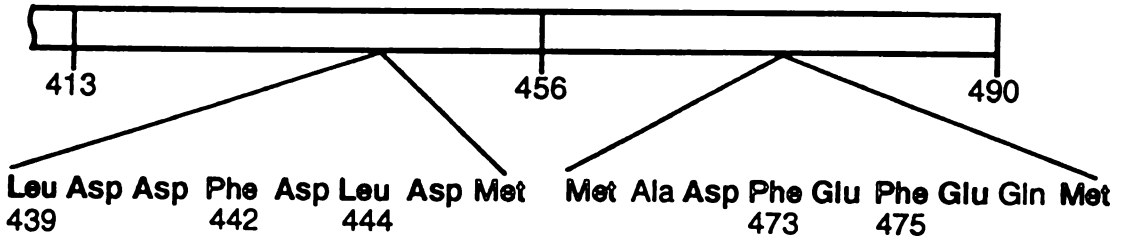

Figure 8. Schematic representation of the VP16 activation domain (amino acids 413-490). The truncated VP16 activation domain (del456) lacks residues 457-490. Portions of the amino acid sequence are shown, using hollow type for hydrophobic amino acids and bold type for acidic amino acids.

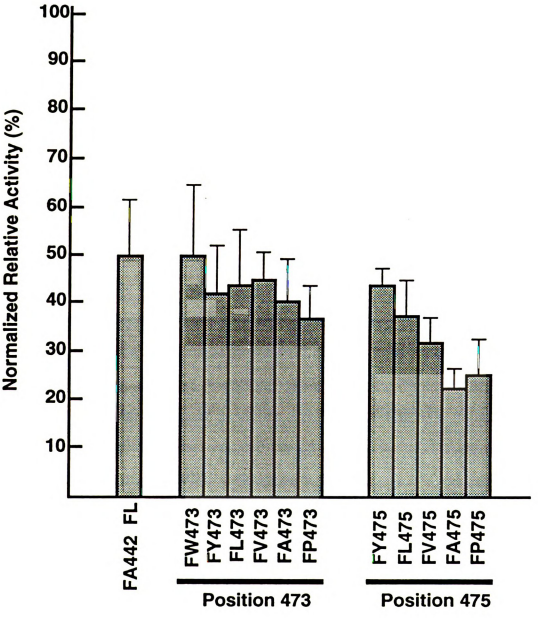

Figure 9. Effects of amino acid substitutions at Phe473 and Phe475 of VP16, tested in the context of the FA442 mutation. Rectangles indicate mean relative activities (normalized to full-length wild type VP16) calculated from at least five independent transfections; error bars represent one standard deviation. FA442 FL represents the activity of the FA442 mutation in full-length VP16 (taken from Figure 7).

the 473 region differ qualitatively in their effect on VP16 activity. We conclude from these results that the proximal and distal subdomains of VP16 have distinct structural features dependent on different patterns of amino acids.

Conclusions

Using oligonucleotide-directed mutagenesis and transient transfection assays, we have analyzed the effects of amino acid substitutions on the activities of both truncated and full-length VP16 activation domains. In the truncated (del456) domain, we have now mutated residue Phe442 to near saturation. Aromatic amino acids were the best substitutes for Phe442, decreasing activity to approximately 30% of wild-type del456 activity. Other hydrophobic substitutions reduced activity to between 10% and 15% of wild-type, while all other substitutions reduced activity to no more than 10% of wild-type activity. Limited mutational analysis of leucine codons 439 and 444 revealed that, although these positions are somewhat less sensitive to mutation, hydrophobic residues at these positions also contribute to the activity of the truncated VP16 activation domain. Thus, the pattern of bulky hydrophobic residues previously observed (Cress and Triezenberg, 1991a) among a variety of transcriptional activation domains has accurately predicted the importance of such residues in the truncated VP16 domain. Whether this pattern has predictive value for other activation domains is presently being tested. Replacement of Trp454 with alanine in the activation domain of the EBNA-2 protein from Epstein-Barr virus reduced the activity of this domain by approximately 80% (Cohen, 1992); in the sequence alignment of Cress and Triezenberg (1991a), Trp454 aligns with Phe442. A portion (aa 564-587) of the activation domain of Rta, another transcriptional

activator protein from Epstein-Barr virus, also fits the Cress and Triezenberg pattern. Simultaneous substitution of glycines for the hydrophobic residues L578, F581, and L582 of Rta (corresponding to L439, F442, and L444 of VP16, respectively) reduced activity of the Rta activation domain by 80 to 90% (Hardwick et al., 1992). Thus it appears that the pattern of hydrophobic and aromatic amino acids observed by Cress and Triezenberg can accurately predict important residues in the activation domains of another herpesvirus transcriptional activator protein.

A partial restoration of transcriptional activity of previously inactive Phe 442 mutants was achieved by adding back residues 457-490 of the VP16 activation domain. The activity of the full-length protein reflected the activity of its truncated parent. For example, FA442 del456 was approximately 15% active relative to wildtype del456, and full-length FA442 was approximately 45% active; FY442 del456 was approximately 35% active relative to wildtype del456, and full-length FW442 was approximately 70% active relative to the activity of full-length VP16.

An examination of the amino acid sequence of residues 457-490 revealed a pattern similar to that surrounding Phe442, with a phenylalanine at position 473 corresponding to Phe442. However, substitutions at Phe473 were not nearly as deleterious to the activity of the distal subdomain as similar substitutions at Phe442 were to the activity of the proximal subdomain. Mutations at Phe475 had a somewhat greater effect on the activity of the distal subdomain. Therefore, the pattern of bulky hydrophobic residues observed by Cress and Triezenberg, which accurately predicted the importance of two leucines flanking Phe442 to the activity of VP16 del456 (the proximal subdomain), was not sufficient in predicting residues in the distal subdomain important for its function. This suggested that the two subdomains might

activate transcription through distinct mechanisms.

The results described here strengthen our previous arguments against prior hypotheses about structural features of acidic activation domains. On one hand, VP16 seems not to fit the "acid blob or negative noodle" hypothesis (Sigler, 1988), which suggests that the activity of such domains is primarily a function of net negative charge (Gill and Ptashne, 1987; Ma and Ptashne, 1987a). Numerous mutations of VP16 at Phe442 have no effect on net charge, and yet they have dramatic effects on transcriptional activation. Furthermore, increasing the net negative charge by replacing Phe442 with Asp or Glu had detrimental rather than beneficial effects on VP16 activity. Apparently, having an aromatic or hydrophobic residue at position 442 is of greater importance than is the net charge of the proximal subdomain.

On the other hand, our results are also inconsistent with a model of VP16 as an amphipathic alpha helix (Giniger and Ptashne, 1987; Zhu et al., 1990). We have previously shown that introducing two potentially helix-breaking proline residues at positions 432 and 436 in the predicted helix of truncated VP16 had no effect on transcriptional activation (Cress and Triezenberg, 1991a), suggesting that the predicted helix (if it does exist) is not necessary for VP16 function. Here, we show the deleterious effect of replacing Phe442 with any of a number of residues predicted to maintain an amphipathic alpha helix, suggesting further that such a structure is not sufficient for activity of the proximal subdomain. These conclusions from our mutational analysis are reinforced by recent spectroscopic studies of the VP16, GCN4, and GAL4 activation domains (Donaldson and Capone, 1992; Van Hoy et al., 1992; Van Hoy et al., 1993) that found little evidence of helical structure. In fact, the GCN4 and GAL4 activation domains appear to form β-sheets (Van Hoy et al., 1992; Van Hoy et al., 1993).

Although our mutations in the truncated VP16 activation domain indicate that an aromatic amino acid is strongly preferred at position 442, we do not yet understand the reason for this preference. The two simplest ideas for the role of Phe442 are either that it is necessary for maintaining the structure of the domain or that it is directly involved in interactions with target proteins in the activation mechanism. Likewise, the reason for the preference for hydrophobic amino acids at positions 439 and 444 is also unknown. It is possible that the combination of hydrophobic residues at positions 439 and 444 is important in correctly positioning Phe442 for making contact with its target. These questions can be addressed by probing the structure of VP16, using spectroscopic or crystallographic methods, and by exploring the association of VP16 with putative target proteins.

In addition to the analysis of the truncated activation domain, we have begun to examine the role of the extreme carboxyl-terminal or distal subdomain of VP16 (aa 457-490). Adding this subdomain onto defective Phe442 mutants partially restored transcriptional activity (Figure 7). Intriguingly, a pattern of acidic and bulky hydrophobic amino acids surrounding Phe473 in this subdomain strikingly resembles the pattern surrounding Phe442 (Figure 8). Furthermore, insertion of four amino acids at codon 471 reportedly abolished transcriptional activity of VP16 (Werstuck and Capone, 1989). However, the types of amino acid substitutions that significantly affected the activity of the truncated activation domain had quantitatively different effects upon the distal subdomain. Specifically, Phe473 (best aligned with Phe442) was relatively insensitive to mutations, whereas Phe475 (best aligned with Leu444) was somewhat more sensitive. These results imply that the pattern of acidic and hydrophobic amino acids, although useful in predicting important residues surrounding Phe442, does

CHAPTER III

RANDOM MUTAGENESIS OF THE VP16 TRANSCRIPTIONAL ACTIVATION DOMAIN

Introduction

The results of the site-directed mutagenesis work described in Chapter II suggested that the VP16 transcriptional activation domain is composed of two subdomains, designated proximal and distal. However, the kinds of mutations that inactivated the proximal subdomain had only modest effects on the activity of the distal subdomain. This suggested to us that the proximal and distal subdomains activate transcription by different mechanisms. Because we lacked clues for targeting specific residues in the distal subdomain for mutagenesis, we decided to randomly mutate the VP16 activation domain and employ a biological selection in yeast to select for mutations in the VP16 activation domain that decrease its function. Using nitrous acid as the mutagen, several missense mutations in the VP16 activation domain were detected that decreased its activity; four mutants had substitutions in the distal subdomain (TA458/DN461/MV478, TA480, IV485, and YC465). The effects of these mutations on the activity of the distal subdomain were tested by determining the abilities of these mutants to activate transcription of a reporter gene; three of the mutations (TA458/DN461/MV478, IV485, and YC465) significantly reduced the activity of the distal subdomain, while the fourth mutation (TA480) had less of an effect.

not correctly describe key residues in the distal subdomain. It is possible that the sequence pattern observed by Cress and Triezenberg may still be predictive of important amino acids in other activation domains that activate by the same mechanism as the proximal subdomain of VP16.

Although secondary structure prediction algorithms suggest that residues 468-478 might fold into an amphipathic alpha helix, our mutational analysis argues against this notion. First, a proline substitution at Phe473 had little effect on the apparent activity of the distal subdomain. Second, the replacement of Phe475 with Ala should be compatible with an amphipathic helix, and yet it had the greatest effect on the activity of this subdomain. However, it is unclear from these results whether the net negative charge of the distal subdomain is a primary determinant of the activity of that domain, since no mutations altering charge were made. We conclude that the proximal and distal subdomains both contribute to the overall activity of VP16, but the structural features necessary for the function of the two subdomains must differ considerably.

A difference in structural features between the proximal and distal subdomains implies that they might activate transcription through different mechanisms. Thus the information gained from the mutagenesis of the proximal subdomain is not likely to be applicable to the distal subdomain, and we were left with no real clues as to which amino acids in the distal subdomain are important for its function. We therefore decided to attempt to identify important residues in the distal subdomain by means of a genetic selection.

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Biological Selection in Yeast

An overarching theme evident from the results of the site-directed mutagenesis of the VP16 activation domain described in Chapter II is that our ability to identify potentially important amino acids using sequence alignments is very limited. Thus, in collaboration with L. Guarente's laboratory at MIT, we devised a biological selection to identify important amino acid changes from randomly mutated VP16 activation domains. Our system takes advantage of the observation that the GAL4-VP16 fusion protein, when present at high concentration, inhibits the growth of yeast, resulting in very small colonies (Berger et al., 1992). This phenomenon has been referred to by some researchers as "squelching" (Gill and Ptashne, 1988). GAL4-VP16 consists of the DNA-binding domain of the yeast transcriptional activator protein GAL4 (aa 1-147) fused to the VP16 activation domain (aa 411-490). Interestingly, VP16 activation domains which are partially deficient in activity are also less inhibitory of yeast growth. In fact, the abilities of certain VP16 activation domains to inhibit growth correlates quite well with the activities of these mutants as determined in our transient transfection assays (Berger et al., 1992).

The strategy, illustrated in Figure 10, is to randomly mutate the VP16 activation domain, and then clone the resulting collection of domains downstream of the GAL4 DNA-binding domain. The GAL4-VP16 genes are on a high copy number (2 micron) yeast plasmid, and expressed constitutively from the strong ADH1 promoter. Yeast are transformed with these constructs by the lithium acetate procedure, and then plated out on media which selects for transformants. The growth of yeast bearing GAL4-VP16 proteins which have fully active VP16 activation domains should be strongly inhibited. Conversely, GAL4-VP16 proteins in which the VP16 activation domain is

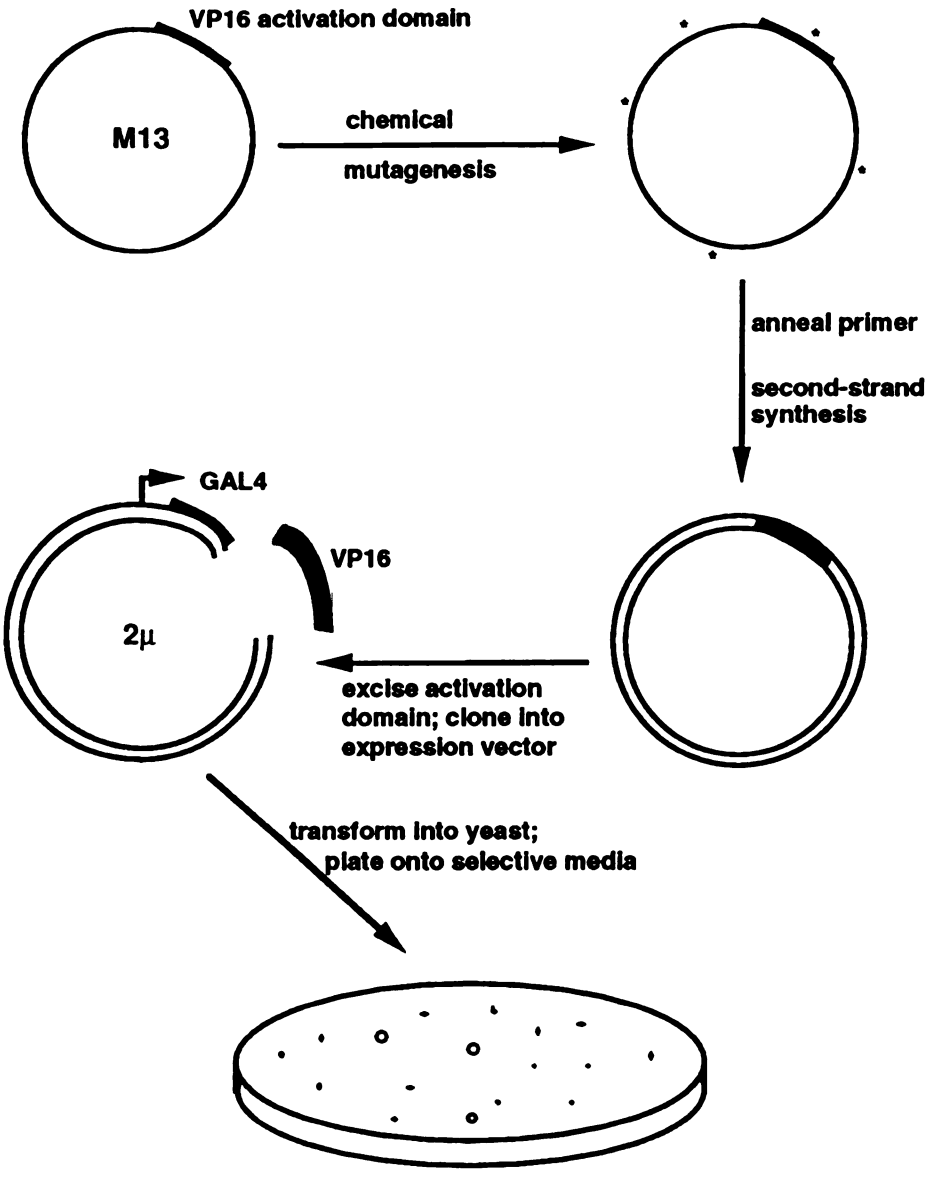

Figure 10. Strategy for chemical mutagenesis and subsequent biological selection in yeast.

significantly less active should be less inhibitory of the growth of the host yeast. Thus, we have a positive growth selection for defective VP16 activation domains.

The names and salient features of the plasmids used in the yeast experiments are described in Table 3. The GAL4-VP16-expressing plasmid pSB201, containing both promoter and terminator sequences from the yeast ADH1 gene and the GAL4 DNA binding domain (aa 1-147), was modified to easily accept DNA fragments encoding mutated VP16 activation domains. After mutagenesis of VP16 activation domains, the resulting collection of restriction fragments was cloned into the modified expression vector (designated ypJR92) in a directed, one step ligation reaction. Modification of pSB201 also allowed easy removal and transfer of mutagenized VP16 activation domains to a new yeast vector; this is an important control to ensure that it is a mutation in the VP16 activation domain, and not in some other part of the expression vector, which gives rise to the large colony phenotype.

Once mutations in the VP16 activation domain had been identified, the transcriptional activity of each mutant was quantitated by cloning the gene for the mutant GAL4-VP16 into the low copy expression vector pSB202delSma, and then assaying the abilities of the mutants to activate expression of a reporter gene, β-galactosidase.

Chemical Mutagenesis

Chemical mutagenesis was employed initially as a method of modifying DNA. The mutagenesis substrate mpJR92, consisting of single-stranded M13 DNA containing the full-length VP16 activation domain, was exposed to two chemicals which modify bases in single-stranded DNA without breaking the

Plasmid Name:	Features:
pDB20LBglII	2 micron (high copy) origin of replication; LEU2 selectable marker; ADH1 promoter and terminator, in between which is a unique BglII cloning site.
pDB20.1	The same as pDB20LBglII, except that a SacI site at position 25 has been eliminated.
ypJR92	Contains a BamHI/BglII fragment encoding GAL4-VP16 cloned into the BglII site of pDB20.1. Used for in vivo toxicity assay.
mpJR92	Contains the sequence of the full-length VP16 activation domain cloned as a SalI/BamHI fragment into M13mp19. Also contains a unique BglII site 3' of the BamHI site. Used as the substrate for chemical mutagenesis.
pSB202	An ARS/CEN (low copy) plasmid; LEU2 selectable marker; ADH1 promoter driving expression of GAL4-VP16. Used for β-galactosidase assay.
pSB202delSma	The same as pSB202, except that VP16 codons 411-452 have been deleted.
pLGSD5	2 micron (high copy) origin of replication; URA3 selectable marker; contains the gene for β-galactosidase driven by the CYC1 promoter and regulated by UAS_G. Used as the reporter for β-galactosidase assay.

Table 3. Description of plasmids used in the yeast biological selection and β-galactosidase assay.

phosphodiester backbone (Myers et al., 1985; Sambrook et al., 1989; Todo et al., 1990). Nitrous acid deaminates deoxycytidine, deoxyadenosine, and deoxyguanosine, changing these nucleosides to deoxyuridine, deoxyinosine, and deoxyxanthidine, respectively. Formic acid acts by breaking the bonds joining purine bases to deoxyribose, thereby depurinating DNA (Myers et al., 1985). Together these chemicals can potentially damage three out of the four bases at a known frequency at specific concentrations and incubation periods.

A potential disadvantage of chemical mutagenesis combined with our biological screen is that only those mutations which have a significant effect on VP16 activity will be identified. Therefore, we will miss mutations that have no effect on activity, which can also be informative. In addition, the cloning strategy subsequent to the chemical mutagenesis to transfer the chemically treated VP16 activation domains into the expression vector ypJR92 utilizes a convenient restriction site (SacI, at codon 424) that is located within the VP16 activation domain; thus, mutations 5' to the SacI site that might reduce VP16 function would not be detected. We felt that such mutations were unlikely, since previous deletion analyses showed that removing codons 413 to 429 of the VP16 activation domain had no effect on function (Triezenberg et al., 1988b).

Two other questions concerning chemical mutagenesis are the efficiency and the distribution of base changes. Based on efficiencies reported in the literature (Myers et al., 1985), we calculated that the chemical mutagenesis described here would be efficient enough so that we could be reasonably confident that the VP16 activation domain target (approximately 250 nucleotides) would suffer frequent damage. Changing incubation times and mutagen concentrations can increase the mutagenesis frequency if that is required. If large colonies were not observed with this mutagenesis protocol,

we can sequence mutagenized activation domains directly to see if the mutagenesis frequency is what we expect.

Since the target DNA is single stranded, and these chemicals are single-strand specific, any potential secondary structure might limit the distribution of base changes. Again, even if certain regions of the VP16 activation domain are damaged less frequently than others, those regions which are damaged and result in the large colony phenotype are of interest. However, if there are regions which consistently escape damage, or if there is a particular region about which we obtain little information, a second mutagenesis method may be employed. One such method utilizes a series of degenerate oligonucleotides which together span the entire VP16 activation domain (Hill et al., 1986). This method allows a great deal of control over the frequency and location of mutations. The oligonucleotides are designed so that each has on average one mismatch with the template DNA. A second method would be to use a modified polymerase chain reaction, increasing the concentration of $MnCl_2$ in the reaction and thereby increasing the misincorporation frequency of Taq polymerase (Leung et al., 1989).

Once the chemical mutagenesis had been performed and the chemicals removed, a primer was annealed downstream of the VP16 activation domains and a complementary DNA strand synthesized using avian myeloblastoma virus reverse transcriptase. This polymerase was used because it is not inhibited by depurinated template DNA (Myers et al., 1985). When reverse transcriptase encounters a damaged base in the template strand, it incorporates nucleotides essentially at random, resulting in a 75% chance of mutation at each damaged site (Sambrook et al., 1989).

After second-strand synthesis, mutated VP16 activation domains were excised from mpJR92 and cloned into the plasmid ypJR92. The resulting

collection of GAL4-VP16-expressing plasmids was amplified in E. coli and then purified by cesium chloride ultracentrifugation before transformation into yeast. We added this step to the published protocol (Myers, 1989) because initially, when the ligation reactions were used to transform yeast directly, many of the resulting transformants exhibited the large colony phenotype but did contain plasmids of the correct size. We deduced that partial (linear) ligation products transformed directly into yeast underwent recombination by the recipient cell, which kept only the selectable marker. Amplification in E. coli avoided this problem, and the majority of large yeast colonies contained plasmids with the activation domain.

The amplified vectors were then transformed into the yeast strain BP1, which lacks endogenous GAL4. The majority of the transformants gave tiny colonies, due to toxicity by fully active GAL4-VP16. The few larger colonies were picked and re-streaked onto fresh selective medium. Yeast colonies that grew when re-streaked were grown in selective liquid medium, and plasmid was harvested. The resulting plasmids were electroporated into E. coli and then plated out onto medium containing ampicillin. Bacterial transformants were picked and grown, and the sizes of the VP16 activation domains were determined by restriction digestion and agarose gel electrophoresis. Correct sized activation domains were recovered from the gel, again cloned into ypJR92, and transformed into BP1 to see if the large-colony phenotype persisted. This re-cloning and re-transformation was done to ensure that the large-colony phenotype was due to mutation in the VP16 activation domain and not elsewhere in the expression plasmid or in the host cell. If the phenotype persisted, the particular yeast expression vector was purified and the DNA fragment encoding the mutated VP16 activation domain sequenced to determine the location and character of the mutation.

Chapter III Methods

Yeast plasmid construction

For in vivo toxicity assays, wild-type or mutant GAL4-VP16 fusion proteins were expressed from the yeast plasmid ypJR92. This plasmid was derived from the plasmid pDB20LBglII (Berger et al., 1992), which is a 2 micron, Amp^r plasmid with a unique BglII restriction site between the alcohol dehydrogenase (ADH) promoter and terminator; it also contains the LEU2 gene, which encodes β-isopropyl malate dehydrogenase, the third enzyme in the leucine biosynthetic pathway. The unique SacI restriction site of pDB20LBglII was destroyed by restriction digestion, filling in, and re-ligation, generating plasmid pDB20.1.

The plasmid pJR3.β58 (Berger et al., 1992) served as the source of the GAL4-VP16 gene. This plasmid is derived from pEMBL 19+ (Triezenberg et al., 1988a) and has the gene for GAL4 (aa 1-147)-VP16 (aa 413-490), plus 400 bp of HSV-1 DNA from the tk gene 3' untranslated region, cloned into the SphI site. To facilitate the cloning of the mutated VP16 activation domains into the yeast expression vector ypJR92, I introduced a unique BglII site at the 3' end of the GAL4-VP16 gene by the following strategy. pJR3.β58 was partially digested with BamHI, which cuts at both the 5' and 3' ends of the GAL4-VP16 gene. Linear DNA fragments from the BamHI partial digest were gel purified and ligated with kinased BglII linkers; ligation products were digested with BglII and then recircularized. The recircularized DNAs were transformed into E. coli, and the colonies which resulted were grown and plasmid harvested. To screen for plasmids that contained the BglII linker, the plasmids were digested with BglII. Plasmids containing a BglII site were then digested with BamHI and ClaI to determine the location of the BglII site. A BamHI/BglII fragment in the orientation of BamHI-GAL4-VP16 gene-BglII

was cloned into the BglII site of pDB20.1; clones containing the GAL4-VP16 gene in the correct orientation relative to the ADH1 promoter were designated ypJR92.

To generate a single-stranded substrate for chemical mutagenesis, the VP16 activation domain (aa 412-490, plus 7 nucleotides downstream of the stop codon) was cloned as a SalI/BamHI fragment into SalI/BamHI-cut M13mp19, generating mpJR91. A BglII site was introduced adjacent to the BamHI site by digesting mpJR91 with BamHI and BglI and cloning in a BamHI/BglI fragment from pEMBL 19/BglII (S. J. Triezenberg, personal communication), thus generating mpJR92.

Chemical mutagenesis of single-stranded DNA

Chemical mutagenesis was performed using procedures described by Myers (1989). Forty µl of 1 µg/µl single-stranded mpJR92 was treated either with formic acid (60 µl of 18 M formic acid, 10 minutes at room temperature) or nitrous acid (10 µl of 2.5 M sodium acetate, pH 4.3, and 50 µl of 2 M sodium nitrite, 60 minutes at room temperature). After treatment with the chemicals, to each reaction was added 100 µl of 2.5 M sodium acetate, pH 5.5, 200 µl of water, 3 µl of 10 mg/ml carrier tRNA, and 1 ml of 100% ethanol. Samples were chilled in a dry ice-ethanol bath, and then spun for 10 minutes in a microcentrifuge. The ethanol wash/precipitation was repeated twice; the chemically treated DNAs were each resuspended in 80 µl TE.

Second-strand synthesis and cloning

To each sample of chemically treated DNA was added 10 µl of 10X reverse transcription buffer (0.5 M Tris-Cl, pH 8.2, 50 mM $MgCl_2$, 50 mM DTT, 0.5 M KCl, 0.5 mg/ml BSA) and 100 pmole of the -20 universal sequencing primer for M13mp19 (New England Biolabs, product #1221). Samples were heated for 5 minutes at 85°C, then 15 minutes at 40°C. To each sample was

added 10 µl of 4dNTP mix (2.5 mM each dATP, dCTP, dGTP, and dTTP) and 40 units of AMV reverse transcriptase (Life Sciences, Inc.); the samples were incubated for 1 hour at 37°C. The extension reaction was stopped by adding 11 µl of 3 M sodium acetate, pH 6, and extracting once with phenol/chloroform, and the DNA was precipitated with ethanol. The DNA was resuspended in TE and then digested with SacI and BglII. The mutated activation domains (250 bp) were purified by agarose gel electrophoresis, and the DNA recovered from the agarose using DNA-affinity resin (Qiagen). Gel-purified mutated activation domains were ligated into SacI/BglII-cut ypJR92, and then transformed into competent HB101 E. coli cells by electroporation (Bio-Rad Gene Pulser, with Bio-Rad 0.2 cm cuvettes). Electroporated HB101 cultures were grown for 1 hour in 1 ml of SOC broth at 37°C and then diluted to 50 ml with LB plus ampicillin. The cultures were grown overnight at 37°C with shaking; the following day plasmid DNA was harvested by alkaline lysis followed by CsCl gradient ultracentrifugation.

Transformation of yeast

Yeast strain BP1 (MATa, ura3-52, leu2-3, ada1-100, GAL4::HIS4) was transformed using the lithium acetate procedure (Becker and Guarente, 1991). A 100 ml culture of BP1 was grown at 30°C with shaking to A_{600} = 0.8. The cells were pelleted and then resuspended in a total of 5 ml of TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM Na_2EDTA, pH 8.0). The cells were pelleted as before and then resuspended in 5 ml of TE/0.1 M lithium acetate. The cells were again pelleted and resuspended in 1 ml of TE/0.1 M lithium acetate. The cells were incubated at 30°C with shaking for 1 hour. After incubation, 100 µl of 4 mg/ml salmon sperm DNA (in TE) was added. Each transformation reaction consisted of 100 µl of competent BP1 cells and 2 µg of yeast plasmid DNA. Samples were incubated for 30 minutes at 30°C without

shaking. After the incubation, 700 µl of 35% (w/v) PEG (MW 3350)/TE/0.1 M lithium acetate was added to each sample, and the samples were mixed by vortexing. The samples were incubated for 50 minutes at 30°C without shaking, and then heat-shocked for 5 minutes at 42°C. After the heat-shock, the cells were pelleted with a 4 second spin in a microcentrifuge. Pelleted cells were resuspended in 500 µl TE; this step was repeated once. Finally, cells were pelleted, resuspended in 100 µl TE, and spread onto Leu$^-$ synthetic complete medium plates to select for transformants. The plates were incubated for 2-3 days at 30°C until colonies appeared.

Recovery of plasmid DNA from yeast

Large yeast colonies were used to inoculate 2 ml of Leu$^-$ synthetic complete liquid medium and grown overnight at 30°C with shaking. The following day, 1.5 ml of each yeast culture was briefly spun to pellet the cells, which were resuspended in 200 µl of yeast lysis buffer (10 mM Tris-Cl, pH 8, 1 mM EDTA, 100 mM NaCl, 0.1% SDS). Glass beads (0.5 mm diameter, Biospec Products), which had been etched with 1 M HCl, were added to the yeast solution to the level of the meniscus. Two hundred µl of phenol/chloroform (1:1; TE saturated) was added to each sample; the samples were vortexed for one minute and then spun to separate the aqueous and organic phases. The aqueous layer from each sample was transferred to a fresh 1.5 ml microfuge tube, 15 µl of 3 M sodium acetate and two volumes of 100% ethanol was added, and the DNA was recovered.

β-galactosidase assay

ypJR92 plasmids containing mutations in the VP16 activation domain were digested with SmaI and the 650 bp SmaI/SmaI fragment was purified by agarose gel electrophoresis and DNA-affinity resin (Qiagen). The recovered fragments were ligated to the 8000 bp SmaI/SmaI fragment of

pSB202delSma1, an ARS/CEN (low copy) yeast vector expressing GAL4-VP16delSma from the ADH1 promoter. The ligation products were screened for the presence of mutant delSma activation domains by digestion with BglII, the restriction site for which is present in mutant delSma activation domains and absent in wildtype delSma activation domains. The orientations of mutant delSma activation domains were determined by DNA sequencing.

Yeast strain BP1 was cotransformed with 2 μg each of pSB202delSma1 containing a mutated VP16 activation domain (amino acids 453-490) and the reporter plasmid pLGSD5 (Guarente et al., 1982). pSB202delSma1 contains the LEU2 gene, and pLGSD5 contains the URA3 gene which encodes orotidine 5'-phosphate decarboxylase, an enzyme required for uracil biosynthesis. The β-galactosidase assays were performed according to Rose et al. (1990). Five ml cultures of cotransformants were grown overnight under selective conditions to A_{600} = 0.8-1.0. The cells were pelleted and the pellets were each resuspended in 250 μl of breaking buffer (100 mM Tris-Cl, pH 7.5, 1 mM DTT, 20% glycerol). Etched glass beads (0.5 mm) were added to the level of the meniscus; then to each sample was added 12.5 μl of 40 mM PMSF (in 100% isopropanol; Boehringer Mannheim). The samples were vortexed six times each at top speed in 15 second bursts, chilling on ice between bursts. Another 250 μl of breaking buffer was added to each sample, and the liquid extracts removed and transferred to fresh 1.5 ml microfuge tubes. The samples were centrifuged for 15 minutes in a horizontal microcentrifuge to clarify; 100 μl of each sample lysate was added to 900 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 30 mM 2-mercaptoethanol; solution pH 7). The samples were incubated at 30°C for 5 minutes. After incubation, to each tube was added 200 μl of 4 mg/ml o-nitrophenyl-β-D-galactoside (ONPG;

Sigma), in Z buffer. Reactions were incubated at 30°C until samples were moderately yellow (usually less than 5 minutes), and then stopped by addition of 500 µl of 1 M Na_2CO_3; the length of reaction time was recorded. The A_{420} of the samples was read. The total protein concentration of each sample was determined using the Bradford assay and absorbance at 595 nm. The specific activities were calculated by the following formula: Activity = $(A_{420})(1.7)/[(0.0045)(\text{protein concentration})(\text{extract volume})(\text{time})]$. The factor 1.7 corrects for the reaction volume, the factor 0.0045 is the optical density of a 1 nmole/ml solution of o-nitrophenol, the protein concentration is in mg/ml, the extract volume is in ml, and the time is in minutes (Rose et al., 1990).

Results

The initial attempt at chemical mutagenesis involved formic acid. The mutagenesis with formic acid was performed as described in the Methods section for this chapter. After cloning of the population of mutated VP16 activation domains into the expression vector ypJR92 and transformation into yeast strain BP1, large yeast colonies were observed. These colonies were picked and restreaked onto fresh minimal medium plates. Those colonies which still exhibited better growth than colonies transformed with unmutated ypJR92 were grown up and their plasmids recovered.

The VP16 activation domains from these large colony mutants were cloned into fresh ypJR92 and then transformed into yeast. Fifteen out of 18 activation domains that gave a large colony phenotype initially also gave the same phenotype after recloning into fresh expression vector. Because few mutations other than in the VP16 activation domain were detected, I omitted the recloning step from the subsequent experiments and sequenced the VP16

activation domains directly from clones exhibiting a large colony phenotype. The following is a summary of the mutations in the VP16 activation domain resulting from treatment with formic acid.

Formic acid treatment resulted in one mutation per activation domain sequenced. The most prevalent mutation (observed in 13 large colonies) was in the natural VP16 stop codon, changing it to the codon for leucine (or, less frequently, tyrosine). This mutation resulted in the addition of 23 extra amino acids to the GAL4-VP16 protein before the next in-frame stop codon. The other mutations were either additions or deletions of bases. The addition of an extra cytosine in a run of six cytosines at VP16 codons 458-460 caused a frameshift which created a new stop codon 15 codons later; this mutation occurred in seven large colonies. All the other frameshift mutations (a total of seven large colonies) were due to a deletion of single bases in the region of VP16 codons 451-463; in addition to the altered coding sequence, these base deletions resulted in at least 40 extra amino acids at the carboxyl end of the GAL4-VP16 protein.

Since formic acid as a mutagen generated many base additions or deletions and few transitions or transversions, I switched to nitrous acid as the mutagen. The mutagenesis using nitrous acid and subsequent biological selection in yeast are described in the Methods section for this chapter. A conscious effort was made to pick yeast colonies that were larger than those transformed with wild type GAL4-VP16 but were not as large as those transformed with the expression vector only. The reasoning was that those colonies which grew as well as the vector only transformants likely had suffered some gross damage to the VP16 activation domain (due to a frameshift mutation, for example), while we were interested in more subtle changes. Larger colonies were picked and then restreaked onto selective

media; those clones which continued to grow better than colonies transformed with wild type GAL4-VP16-expressing plasmids were analyzed further.

Approximately 150 larger yeast colonies were picked and restreaked; greater than 80% of the clones continued to grow better than wild type GAL4-VP16 transformants. The sizes of the VP16 activation domains of 53 of these clones that grew when restreaked were determined by restriction digest and electrophoresis. Nineteen clones had activation domains of the correct size and were therefore sequenced. Of the nineteen VP16 activation domains sequenced, six had no apparent mutation in the activation domain; presumably the large colony phenotype was due to host mutations or mutations elsewhere in the expression plasmid. Two clones had mutations that did not alter the amino acid specificity, and two clones had frameshift mutations in the VP16 activation domain (the addition or deletion of a deoxyadenosine at codon 460). The remaining nine clones all had missense mutations, the locations of which are shown in Figure 11 and described below.

Five yeast colonies (N93, N101, N109, N111, and N151) contained identical mutations, a glycine to glutamic acid change at codon 448 and a threonine to alanine change at codon 480. The ambiguity in establishing which mutation caused the large colony phenotype was relieved by the isolation of another large colony mutant (N112) having only the TA480 mutation.

Another yeast colony (N105) contained a VP16 activation domain with four amino acid substitutions, aspartic acid to glycine at codon 427, threonine to alanine at codon 458, aspartic acid to asparagine at codon 461, and methionine to valine at codon 478. Mutant N144 had two mutations, a

Figure 11. Summary of mutations resulting from treatment of the gene encoding the VP16 activation domain with nitrous acid. The amino acid sequence of the VP16 activation domain is shown. The location and type of mutation, along with the identifier (N for the mutagen nitrous acid, the number referring to the original larger yeast colony picked).

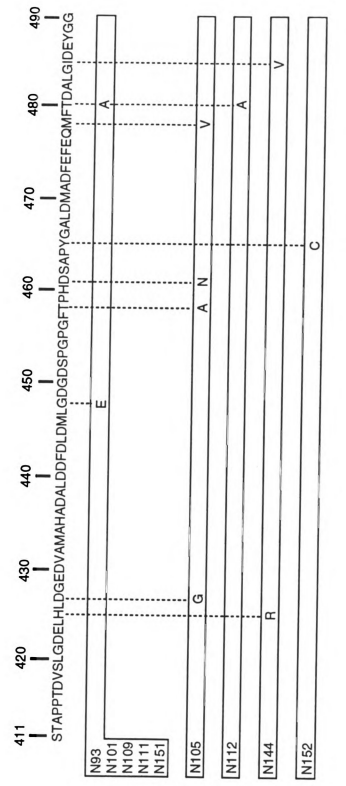
411
420
430
440
450
460
470
480
490
STAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG
N93
N101
N109
N111
N151
N105
N112
N144
N152
E
A
G
A N
V
A
R
V
C

Figure 11

histidine to arginine change at codon 425 and an isoleucine to valine change at codon 485. Interestingly, the HR425 mutation had previously been generated and tested in the context of VP16 del456, and this mutation had no effect on activity (D. Cress, unpublished data). Therefore we inferred that the large colony phenotype was likely due to the IV485 mutation. Finally, mutant N152 had a single amino acid substitution, tyrosine to cysteine at codon 465.

We were especially interested in those mutations which occurred in the distal subdomain of the VP16 activation domain, since our previous site-directed mutagenesis work in the subdomain was uninformative (see Chapter II). Therefore I concentrated my efforts on the clones N105, N112, N144, and N152. The yeast phenotypes conferred by these four GAL4-VP16 mutants are shown in Figure 12.

To quantitate the activities of the four GAL4-VP16 mutants, I performed β-galactosidase assays, which measures the ability of GAL4-VP16 mutants to activate expression of a reporter gene encoding the enzyme beta-galactosidase. For this assay, the various GAL4-VP16 mutant proteins were expressed from a low copy (ARS/CEN) yeast plasmid to minimize the toxic effects caused by overexpression of these proteins. The mutant VP16 activation domains tested consisted only of codons 453-490. This was done because we wished to determine the effect of these mutations on the activity of the distal subdomain alone, since both the site-directed mutagenesis work described in Chapter II and recent collaborative work (described in Chapter IV) indicate that the two subdomains of the VP16 activation domain function independently and through different mechanisms.

The β-galactosidase assay involves growing 5 ml cultures of yeast strains harboring both the plasmid expressing the mutant GAL4-VP16 and the

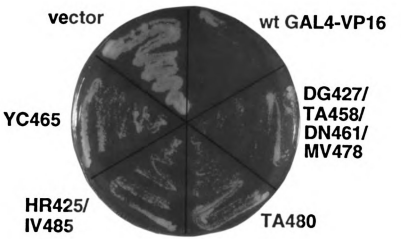

Figure 12. Mutations in the VP16 activation domain of GAL4-VP16 relieve in vivo toxicity. Approximately equal sized yeast colonies containing plasmids expressing either wild-type GAL4-VP16 or mutant derivatives thereof were picked and restreaked onto selective media. The vector only control is plasmid pDB20.1 expressing no GAL4-VP16 protein.

GAL4-VP16-responsive reporter plasmid. This growth is done under selective conditions to ensure that both plasmids are retained. After growth, cells are disrupted by agitation in the presence of glass beads. An aliquot of the resulting cell lysate is mixed with an ONPG solution and incubated at 30°C until the sample turns a pale yellow, after which the reactions are terminated. The specific activity of each sample is a function of the amount of o-nitrophenol produced (determined by absorbance at 420 nm), the reaction time, the volume of cell lysate tested, and the total protein concentration of the cell lysate.

The activities of the four mutants described above relative to both full-length GAL4-VP16 and GAL4-VP16delSma (which lacks the proximal subdomain) are shown in Figure 13. As a control, a full-length mutant with a phenylalanine to alanine substitution at position 442 was also tested. The FA442 mutation decreased activity to approximately 30% of wild-type GAL4-VP16, a value which is in reasonable agreement with published results (Berger et al., 1992). The activity of this mutant is somewhat lower in this assay than what was determined by transient transfection assays (see Figure 7, Chapter II). Deletion of the proximal subdomain of the VP16 activation domain reduced the ability to activate transcription by approximately 40%; however, this result also demonstrates conclusively that the distal subdomain of the VP16 activation domain is sufficient for activating transcription even in the absence of the proximal domain.

Three of the mutations, TA458/DN461/MV478, IV485, and YC465, significantly diminished the activity of the distal subdomain. The TA458/DN461/MV478 mutation reduced activity to approximately one-third of the activity of GAL4-VP16delSma (18% of full-length GAL4-VP16). The IV485 mutation reduced activity to a level similar to that of the

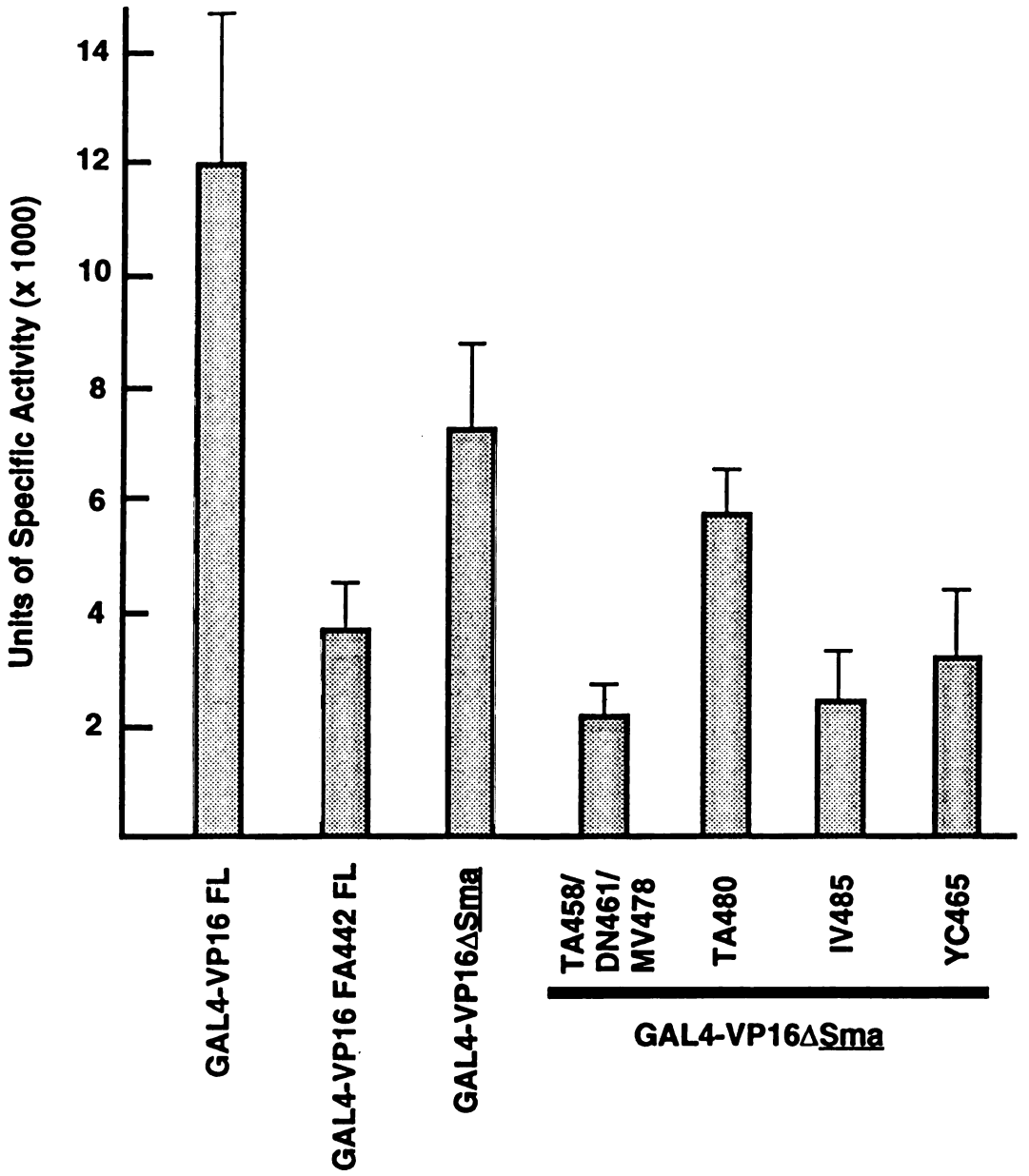

Figure 13. Relative activities of GAL4-VP16delSma proteins bearing mutations in the distal subdomain of the VP16 activation domain. Rectangles indicate the mean relative activities of at least three independent yeast transformants; error bars represent one standard deviation. Activities were corrected for background (no GAL4-VP16).

TA458/DN461/MV478 mutation, to approximately one-third of that of GAL4-VP16delSma and approximately 20% of full-length GAL4-VP16 activity. The YC465 mutation reduced activity to approximately one-half of that of GAL4-VP16delSma (30% of full-length GAL4-VP16 activity).

The fourth mutation, TA480, only reduced activity to a level approximately 10% below that of GAL4-VP16delSma (50% of full-length GAL4-VP16 activity). An examination of Figure 12 does not reveal a significant difference in yeast growth rate between mutant N112 and the other three nitrous acid mutants. It is possible that the TA480 mutation only exerts its effects on activation when it is in the context of the full-length VP16 activation domain.

Conclusions

The site-directed mutagenesis work described in Chapter II suggested that the VP16 activation domain consists of two subdomains, designated proximal (aa 410-456) and distal (aa 457-490), and that these two subdomains activated transcription through different mechanisms. However, those studies, while successful in elucidating amino acids critical for the function of the proximal subdomain, did not reveal which amino acids in the distal subdomain were critical for its function. The observation that overexpression of a GAL4-VP16 fusion protein in yeast was toxic to yeast growth led to the development of a biological selection for mutations in the VP16 activation domain that relieved toxicity.

Chemical mutagenesis using nitrous acid of a DNA fragment encoding the full-length VP16 activation domain, followed by a biological selection in yeast, produced GAL4-VP16 mutants bearing amino acid changes in the VP16 activation domain. Many of the nitrous acid mutants had multiple amino

acid substitutions; in all of the nitrous acid mutants, however, at least one mutation occurred in the distal subdomain.

Four of the nitrous acid mutants, N105 (DG427/TA458/DN461/MV478), N112 (TA480), N144 (HR425/IV485), and N152 (YC465), decreased the activity of GAL4-VP16, thereby partially relieving toxicity. These four mutants were characterized further by cloning the distal subdomain fragment bearing the mutation(s) into a low-copy yeast expression vector and testing their abilities to activate a reporter gene encoding β-galactosidase. Three of the four mutants (N105, N144, and N152) had significantly reduced activities relative to that of GAL4-VP16delSma. The fourth mutant (N112) had only slightly reduced activity relative to that of GAL4-VP16delSma, suggesting that its effects were only exerted in the context of the full-length VP16 activation domain.

The distal subdomain mutations discovered by means of the biological selection in yeast point to certain regions of this subdomain as being important for its function. No deleterious mutations were detected in the region aa 470 to 477 that includes Phe473 and Phe475, the region that was proposed to be important for function by virtue of sequence similarity with the region surrounding Phe442. Instead, a mutation prior to this region (YC465) and three mutations after this region (MV478, TA480, and IV485) appear to decrease the activity of the distal subdomain. The tyrosine to cysteine change at position 465 is somewhat problematic, due to the potential for cysteine to form disulfide bonds; such bonds could potentially cause GAL4-VP16 proteins to multimerize into inactive complexes. A simple way to determine the importance of Y465 for the activity of the distal subdomain would be to make other amino acid substitutions at this position.

The methionine to valine change at position 478 is also difficult to

interpret because it occurs in conjunction with the TA458 and DN461 mutations. These latter two mutations are at the very beginning of the region we have designated the distal subdomain, a region that may be involved in properly positioning the two subdomains relative to one another. The obvious way to avoid this difficulty would be to test the effect of the MV478 mutation alone, or also in conjunction with TA458 or DN461 but not both.

The threonine to alanine change at position 480 was surprising in that its effects were more pronounced in the context of full-length GAL4-VP16 than in GAL4-VP16delSma. However, the activity of this mutation in the full-length context has not been quantitated by β-galactosidase assays, so direct comparisons of this mutation between the two contexts is difficult at this time. The isoleucine to valine change at position 485 showed a more consistent pattern: this single substitution both relieved in vivo toxicity and significantly reduced the activity of the distal subdomain. This mutation is fairly conservative, although valine in less hydrophobic than isoleucine (Nozaki and Tanford, 1971; Von Heijne and Blomberg, 1979). The fractions of time these two amino acids are found buried in the interior of proteins are approximately equal (Chothia, 1976), so it is doubtful that this mutation results in a large conformational change. It is more likely that the shorter side chain of valine is less able to contact the molecular target of the distal subdomain.

CHAPTER IV

MECHANISM OF ACTION OF THE VP16 ACTIVATION DOMAIN

Introduction

In this dissertation I have described various means I have employed to identify amino acids within the VP16 activation domain that are important for the function of that domain. VP16 has in recent years become a standard against which other transcriptional activator proteins are compared, and to date its activation domain is the most thoroughly studied. Therefore our knowledge about VP16 may also enhance the study of other transcriptional activator proteins, as well as deciphering how transcriptional activator proteins fit into the process of gene regulation. In this chapter I will discuss how the various VP16 activation domain mutants characterized in this dissertation have been utilized to unravel the mechanism of action of the VP16 activation domain.

Mechanism of Action of the VP16 Activation Domain

A number of potential mechanisms for transcriptional activation by VP16 are now being investigated. One mechanism may involve a direct interaction of the activation domain with basal transcription factors. For example, the VP16 activation domain has been used as an affinity reagent for chromatography of nuclear extracts, and the basal factor TFIID was preferentially bound (Stringer et al., 1990). Truncation of VP16 at position 456

reduced but did not abolish this interaction; point mutations at Phe442, within the del456 context, bound TFIID in proportion to their transcriptional activities (Stringer et al., 1990; Ingles et al., 1991). However, Lin and Green (Lin and Green, 1991), using a similar strategy, found that VP16 affinity columns retained the basal factor TFIIB more stably than TFIID. The truncated VP16 activation domain (del456) bound TFIIB less well than did full-length VP16; the FP442 mutation greatly diminished binding of TFIIB. Why these two papers reported different results has not been resolved.

The basal transcription factor TFIIH has also been shown recently to be a potential target of the VP16 activation domain (Xiao et al., submitted). These authors report that the VP16 activation domain can bind to TFIIH in the absence of other basal transcription factors (such as TFIID or TFIIB). The truncated VP16 activation domain (del456) was greatly reduced in TFIIH binding; full-length Phe442 mutants also showed reduced binding to TFIIH, although the reduction was slightly less than that resulting from the del456 truncation. It is not possible from these results to definitively assign TFIIH binding function to the proximal or distal subdomains of VP16 because the VP16 delSma activation domain was not tested for its TFIIH-binding ability.

In addition to TFIID, TFIIB, and TFIIH, a fourth potential target molecule for the VP16 activation domain has also been described. Several groups have reported both biochemical and genetic evidence for the existence of factors (termed adaptors, mediators or coactivators) that are required for activated but not basal transcription (Berger et al., 1990; Kelleher III et al., 1990; Pugh and Tjian, 1990; Berger et al., 1992). Berger et al. (1990) demonstrated that a GAL4-VP16 fusion protein consisting of the GAL4 DNA binding domain and the full-length VP16 activation domain, in the presence of a 20 basepair oligonucleotide containing the GAL4 binding site, could inhibit

activated but not basal transcription from a different template. Strikingly, a GAL4-VP16 fusion containing the truncated VP16 activation domain (del456) did not inhibit activated transcription under the same conditions, although it could activate transcription somewhat less effectively than did the full-length GAL4-VP16 fusion. Thus, the distal subdomain may interact with the adaptor, and the proximal subdomain may still retain some capability for interaction with another component such as TFIID, TFIIB, or TFIIH.

The gene encoding the adaptor protein ADA2 has been cloned in yeast, using a strategy very similar to the one I used to identify detrimental mutations in the VP16 activation domain. Berger et al. (1992) isolated mutant yeast colonies that were resistant to the toxic effects resulting from overexpressing GAL4-VP16; the majority of the resistant colonies had mutations in the GAL4-VP16-expressing plasmid, but a few had host mutations; among these yeast mutants three complementation groups (ada1, ada2, and ada3) were identified. The mutation in ada1 reduced expression of GAL4-VP16, whereas the ada2 and ada3 mutations did not.

To clone the wild type ADA2 gene, clones from a yeast genomic library were identified that complemented the ada2 mutation. The ADA2 open reading frame encodes a 434 amino acid protein (ADA2) that exhibits no obvious homology to genes from other organisms. Nuclear extracts from ada2 mutant yeast strains were not able to support activated transcription by GAL4-VP16 in vivo. The yeast activator GCN4 was also unable to activate transcription in ada2 mutant strains. However, the yeast activators HAP1 and HAP2/3/4 were still capable of activating transcription in ada2 mutant strains, suggesting that ADA2 is not used by all acidic activators; this also suggests that there are multiple mechanisms by which yeast activator proteins activate transcription.

The notion that the distal subdomain of VP16 interacts with the adaptor protein, suggested by Berger et al. (1990), has been strengthened by several recent results. Using co-immunoprecipitation, GAL4-VP16delSma but not GAL4-VP16del456 was shown to bind ADA2 (N. Silverman, personal communication). Furthermore, GAL4-VP16delSma is unable to activate transcription in ada2 extracts or cells, whereas GAL4-VP16del456 can still activate transcription in ada2 extracts or cells. GAL4-VP16delSma is also able to squelch transcription from a heterologous promoter in vitro, whereas GAL4-VP16del456 does not (S. Berger, personal communication).

The recent demonstration that the VP16delSma activation domain, when fused to the GAL4 DNA-binding domain, was able to activate transcription from a GAL4-regulated gene (S. Berger, personal communication; Figure 13) contradicts an earlier report in which a deletion mutation that removed amino acids 413-443 of VP16 abolished activity (Triezenberg et al., 1988b). The delSma VP16 activation domain includes ten fewer VP16 amino acids than that used by Triezenberg et al. (1988b), the inclusion of which may cause the distal subdomain to fold improperly.

Finally, a fifth factor has been proposed as a target of the VP16 activation domain (He et al., 1993; Li and Botchan, 1993). DNA replication factor A (RPA) is a trimeric single-stranded DNA-binding protein required for initiation of DNA replication in vitro (So and Downey, 1992). He et al. (1993) show that the VP16 activation domain, when used as an affinity reagent, was able to bind to RPA-1, the largest subunit of RPA, from HeLa cell extracts. The affinity of the VP16 activation domain for RPA-1 was decreased when the truncated (del456) VP16 activation domain was used as the affinity reagent; the FY442 del456 mutation further diminished RPA-binding, while the FP442 del456 mutation abolished RPA-binding almost completely. Thus the

abilities of VP16 activation domain mutants to bind RPA correlate well with their abilities to activate transcription (Cress and Triezenberg, 1991a). The RPA-binding abilities of the VP16 activation domain mutants was also shown to correlate with the ability of GAL4-VP16 proteins bearing these mutations to activate the replication of a plasmid containing the polyomavirus origin of replication in mouse FOP cells (He et al., 1993). These authors suggest that the interaction between VP16 and RPA may stimulate RPA-dependent unwinding by the appropriate cellular DNA helicase; unwinding of the DNA strands is necessary for both transcription and replication, which might explain why a prototypical transcriptional activator protein can also bind to a replication factor.

The obvious question is what is the true biological role of VP16? Or, more specifically, how does VP16 activate transcription? At this time it is not possible to discriminate between the mechanisms described above. It is very likely that VP16 activates transcription through several mechanisms. The data presented in this dissertation and the results from collaborations with the Guarente laboratory strongly suggest that the proximal and distal subdomains of VP16 function independently by different mechanisms.

The GAL4-VP16 protein, when expressed at high levels in yeast, is toxic to the growth of the host yeast. Mutations in either the proximal or distal subdomains of VP16 can partially relieve this toxicity. For example, a phenylalanine to alanine change at position 442 of the full-length GAL4-VP16 protein can reduce toxicity (Berger et al., 1992); likewise an isoleucine to valine change at position 485 of the full-length GAL4-VP16 protein can also reduce toxicity. Results from Berger et al. (1990) and the co-immunoprecipitation data from Neal Silverman suggest that it is the distal subdomain that binds to the adaptor ADA2. The del456 VP16 activation

domain does not appear to bind ADA2, therefore the GAL4-VP16 FA442 (full-length) mutant that partially relieves toxicity must do so through some other mechanism.

Based on our current knowledge I propose the following model (Figure 14). The proximal subdomain of the VP16 activation domain activates transcription by binding to a basal transcription factor, several candidates being TFIID, TFIIB, and TFIIH. The truncated (del456) VP16 activation domain activates transcription solely through this mechanism. The distal subdomain activates transcription by binding to the adaptor protein, which in the case of ADA2 mediates activated transcription by some but not all activators (Berger et al., 1992). The adaptor protein also binds to some component of the basal transcription complex and thereby strengthens the interaction between the activator and the basal transcription complex.

Mutations at Phe442 (or Leu439 and Leu444) in the truncated activation domain reduce the ability of this domain to bind to the basal factor(s). When these proximal subdomain mutations are placed in the context of the full-length VP16 activation domain, the distal subdomain binds to the adaptor, which stabilizes the weakened interaction between the proximal subdomain and its basal transcription factor target(s). The distal subdomain is able to activate transcription independent of the proximal subdomain because the adaptor binds to the basal transcription complex.

It is not known at this time if the mode of action of the VP16 activation domain, which appears to activate transcription by at least two mechanisms, is typical of other transcriptional activators. This model may only be applicable for other transcriptional activation domains that function by the same mechanisms as does VP16, for instance through simultaneous binding of a basal transcription factor and ADA2. The yeast HAP activators appear to

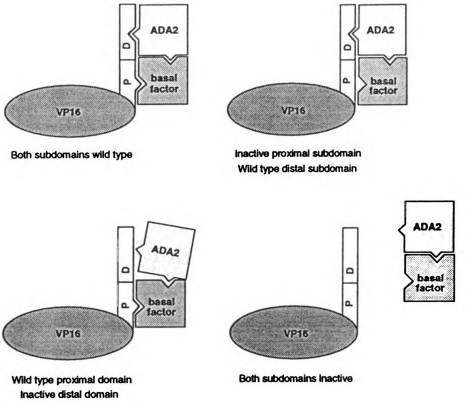

Figure 14. Proposed model of the mechanism of activation by the VP16 activation domain. The letters P and D refer to the proximal and distal subdomains of VP16, respectively. ADA2 mediates transcriptional activation by the VP16 activation domain in yeast.

work through a different adaptor protein (or no adaptor), so this model likely has little predictive value for those activators.

Future Studies

The four distal subdomain mutations that were tested for the ability to activate transcription of a reporter gene each represented only one of the many possible amino acid substitutions at that particular position. This is a consequence of the mutagenesis method that was employed. Because of this limitation we cannot say whether the decrease in function was due to the presence of a different amino acid or the absence of the wild type amino acid; it is likely that other changes at these positions may also decrease activity. Site-directed mutagenesis at these positions will help to explain why the changes reported here had their effects. For example, I speculated in Chapter III that one explanation for the decrease in activity caused by the IV485 mutation could be that the valine side chain is shorter than that of isoleucine. A leucine substitution at this position might be less deleterious to activity, since the side chains of leucine and isoleucine are similar in length. I feel that the most efficient method for testing the activities of additional substitution mutants is by the β-galactosidase assay.

Additional methods of mutating the distal subdomain, combined with the biological selection in yeast, should identify other amino acids important for the function of this subdomain. I believe it is important to explore other methods of mutating the distal subdomain, since the mutants I have isolated are not evenly dispersed across the subdomain and the chemical mutagenesis is not totally random. Peter Horn in our laboratory is currently using the polymerase chain reaction, modified to increase the misincorporation frequency of Taq polymerase, to mutate the distal subdomain. This method is

not biased in the types of mutations it can generate. He will use the same in vivo selection described here to isolate mutations in the VP16 activation domain. Peter will also search for VP16 activation domain mutants which can suppress point mutations in the ADA2 protein.

Since we now have mutations which inactivate either the proximal or distal subdomain, we can generate different combinations of active and inactive subdomains and use them to test my proposed model. For example, my model predicts that VP16 can still activate transcription even if only one of the two subdomains is functional; the combination of inactive proximal and distal subdomains should bind very poorly to both the adaptor and the basal factor(s) and therefore be almost totally non-functional.

My model also suggests that, if the proximal subdomain is toxic in yeast, it should be possible to select for mutations in the target of this subdomain that relieve toxicity, in the same manner that mutations in the gene encoding the adaptor ADA2 were isolated. It has not yet been demonstrated, however, that overexpression of a GAL4-VP16del456 protein is toxic to yeast growth.

It would be interesting to determine if the mutations in the distal subdomain of VP16 that reduce the activity of that subdomain also reduce its activity in mammalian cells. This could easily be tested in the transient expression assay that was described in Chapter II. The transient expression assay might enable us to isolate a mammalian counterpart (if one exists) to the yeast ADA2 protein.

Those VP16 activation domain mutants that decrease its function will also be useful for structural studies that are in progress in our laboratory. Fan Shen will be using analytical ultracentrifugation to determine the approximate shape of GAL4-VP16 proteins containing wild type or mutant VP16 activation domains; some of our mutations presumably alter the

conformation of the VP16 activation domain, which might be detectable in Fan's experiments. Other VP16 mutations might not alter the structure appreciably, and therefore may decrease activity by altering a side chain important for binding to the target protein. Fan will also use analytical ultracentrifugation to determine if the VP16 activation domain (or mutant derivatives thereof) can interact in solution with purified TBP or TFIIB.

Transcriptional activator proteins other than VP16, especially those that appear to activate transcription by similar mechanisms (for instance, via ADA2), should benefit from the information gained from our mutagenesis studies. Knowledge of the similarities and differences between structural features and mechanisms of action of VP16 and other transcriptional activators will increase our understanding of how this class of proteins functions in the cell as one mechanism of gene regulation.

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