VIROLOGY 221, 34–43 (1996)
ARTICLE NO. 0350

Genetic Analysis of the Bovine Papillomavirus E2 Transcriptional Activation Domain

*Department of Dermatology, New England Medical Center and Tufts University School of Medicine, and †Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received February 26, 1996; accepted May 2, 1996

The bovine papillomavirus type 1 E2 transactivator has a large amino-terminal 215-residue transcriptional activation domain (TAD) that is active in *Saccharomyces cerevisiae* and higher eukaryotic cells. Comparison to other transcriptional activators suggests that its functions may be mediated in part through two acidic regions, A1 and A2, in this domain. We have characterized the functional elements within the E2 TAD using LexA–E2 fusions and by screening randomly generated libraries of E2 mutations for transcriptional activation in yeast. The A1 region was highly sensitive to substitutions that reduce negative charge, although there was not a perfect correlation between overall charge and transcriptional activity. Mutations were isolated within a hydrophobic amino acid motif that overlaps the A2 region and resembles elements described in other viral and cellular transactivation domains. When fused to the LexA DNA binding domain, this hydrophobic motif within the acidic A2 region was unable to activate transcription in *S. cerevisiae.* Multiple highly defective mutations primarily altering hydrophobic amino acids were identified in the distal third of the E2 TAD. The transcription phenotype of many of these E2 TAD mutations was similar in yeast and COS cells.

INTRODUCTION

The papillomavirus E2 protein is essential for viral gene expression and replication, processes known to be tightly linked to the differentiation program of the host epithelial cell. One model is that E2 acts as an enhancer by facilitating the assembly of basal transcription complexes which subsequently initiate RNA synthesis. The bovine papillomavirus type 1 (BPV-1) E2 gene encodes a 410-amino-acid protein. The N-terminal 215 residues of E2 are required for transcriptional activation and while the functional elements of this region have not been characterized, it is presumed to coordinate interactions with cellular factors (Dostatni *et al.,* 1988; Giri and Yaniv, 1988; Haugen *et al.,* 1988; McBride *et al.,* 1988, 1989; Moskaluk and Bastia, 1989). The C-terminal 125 amino acids of E2 specifically bind the DNA sequence $ACCGN_4CGGT$, an element found in close juxtaposition to most papillomavirus promoters (Androphy *et al.,* 1987a,b; Moskaluk and Bastia, 1988) which functions as an E2-dependent enhancer (Harrison *et al.,* 1987; Haugen *et al.,* 1987; Spalholz *et al.,* 1987; Hawley-Nelson *et al.,* 1988).

The mechanism by which the N-terminal E2 transcriptional activation region stimulates gene expression has yet to be resolved. E2 has been demonstrated to cooperatively stimulate transcription *in vivo* with the TATA binding protein (TBP) (Steger *et al.,* 1995; Rank and Lambert, 1995), TFIIB (Rank and Lambert, 1995), and cellular transcription factors including Sp1 (Li *et al.,* 1991), USF, and CTF (Ham *et al.,* 1991; Ushikai *et al.,* 1994). The N-terminal half of the E2 TAD has been proposed to form two acidic amphipathic helices (Giri and Yaniv, 1988), a motif common in viral and cellular transcriptional activators. Like many "acidic" activators, E2 transcription functions are detectable in *Saccharomyces cerevisiae* (Lambert *et al.,* 1989; Morrissey *et al.,* 1989; Stanway *et al.,* 1989). Transcriptional activation by E2 is abolished by deletion of the acidic regions, yet it has not been shown that the negatively charged amino acids of this region are responsible for an E2 function.

Genetic analyses of transcriptional activation domains including those from VP16, RTA, and SP1 have identified bulky hydrophobic (BH) amino acid motifs which are critical to function (Cress and Triezenberg, 1991; Hardwick *et al.,* 1992; Regier *et al.,* 1993; Blair *et al.,* 1994; Gill *et al.,* 1994). These motifs are thought to mediate protein–protein interactions with basal transcription factors such as TBP, TFIIB, and TAFs (Gill *et al.,* 1994). Mutation of individual BH residues at consensus positions in some BH motifs, including F443 of VP16, can result in complete inactivation of transactivation function (Cress and Triezenberg, 1991; Regier *et al.,* 1993). We have noted similar motifs in the acidic regions of papillomavirus E2 proteins, but their functional significance is unknown. To identify transcriptionally defective E2 mutants, we performed a genetic screen in *S. cerevisiae* in order to characterize functional domains and amino acids of its TAD.

[1] To whom correspondence and reprint requests should be addressed at Department of Dermatology, New England Medical Center Box 166, 750 Washington Street, Boston, MA 02111. E-mail: eandroph@opal.tufts.edu.

0042-6822/96 $18.00

MATERIALS AND METHODS

PCR misincorporation mutagenesis was used to create three mutant libraries targeting the transcriptional activation domain of BPV-1 E2 (A: residues 1–71; B: residues 71–134; and C: residues 133–216) (Leung *et al.,* 1988). Mutant libraries A and B were generated using primer set M1 (5′ CTCGGATCCCATGGAGACAGCATG 3′, nt 2608–2623) and M2 (5′ GCTGCCAGCCGTCCTC 3′, nt 3047–3032). The M1/M2 PCR product was gel purified, cleaved with *Bam*HI/*Bgl*I (library A, nt 2608–2815) or *Bgl*I/*Acc*I (library B, nt 2818–3006), and ligated separately to M13mp19E2-286 which was cleaved with the same enzymes. Library C used primers M3 (5′ CCAGGGTGGTAGAGGTG 3′, nt 2952–2956) and M4 (5′ CCTTCGCTAGCGACCCAGACTC 3′, nt 3261–3240). Primer M4 introduces a translationally silent *Nhe*I site at nucleotides 3251–3256. The M3/M4 PCR product was cleaved with *Acc*I and *Nhe*I and ligated to similarly cleaved pUC19 E2-286^{N}, which contains the engineered *Nhe*I site. Ligation reactions were introduced into *Escherichia coli* MV1190 (libraries A and B) or DH5α (library C) by electroporation, and recombinant virus or colonies from several plates were pooled. Double-stranded replicative form M13mp19 E2 286 DNA (RF DNA) was prepared after infection of MV1190. The mutant RF DNA or plasmid DNA was cleaved with *Bam*HI and *Kpn*I and the E2 1–286 fragment was purified and ligated to the YEplac112G-E2 fragment encoding E2 amino acids 287–410. YEplac112G, modified from YEplac112 (Gietz and Sugino, 1988), includes the GAL 1-10 UAS with the TRP1 gene and 2μ yeast replicon. These ligation reactions were introduced into DH5α by electroporation and resulted in individual libraries of approximately 25,000 transformants.

E2 mutant libraries were screened in the *S. cerevisiae* strain DBY1. This strain was constructed by inactivation of the TRP1 gene of BGW17a (MATa, leu2, 2-leu2, -11; his4–his519, ade1–-ade100,ura3–ura52) used for previous genetic studies of BPV-1 E2 (Prakash *et al.,* 1992). The TRP1 gene of BGW1-7a was inactivated by one-step gene replacement (Alani *et al.,* 1987). The YEplac112GE2 mutant library was transformed into DBY1 containing pBsy72 (Morrissey *et al.,* 1989). Transformants were selected on minimal medium (YMM) minus tryptophan and uracil supplemented with 2% glucose and subsequently replica plated to galactose/X-gal media (Prakash *et al.,* 1992). After 48 hr at 30° white and light blue colonies on the galactose plate were selected from the corresponding YMM/glucose plate. Putative mutant E2 plasmids were then recovered from these cells by transformation of total DNA extracts into DH5α and reintroduced into DBY1 carrying pBsy 72 to confirm the transactivation phenotype (Ausbel *et al.,* 1993). For immunoblots, the cells were cultured in YMM with galactose for 12 hr, pelleted, and resuspended in 2$\times$ SDS/PAGE sample buffer. Yeast extracts were resolved by polyacrylamide electrophoresis and electroblotted to Immobilon P (Millipore), and the presence of full-length E2 was determined by Western blot with rabbit antiserum to BPV-1 E2 (Androphy *et al.,* 1987a). Mutants which scored positive for synthesis of full-length E2 in the Western blot and which failed to transactivate the reporter after repeat testing were characterized by DNA sequence analysis with Sequenase 2.0 (U.S. Biochemicals).

Fusions of the LexA DNA binding domain to E2 were made using 2μ vectors, YEplac181GLexA202 (LEU2) and YEplac112GLexA202B (TRP1), in which synthesis of the 202-amino-acid LexA DNA binding domain, obtained from pEG202 (Gyuris *et al.,* 1993), was galactose inducible from the GAL 1-10 UAS. Transactivation by LexA–E2 fusions was performed by introducing the fusion into DBY1 along with the LexA/LacZ reporter pSH18-34 (kindly provided by R. Brent). Mutations of E2 were excised from YEplac112GE2 as *Sph*I/*Kpn*I fragments and transferred to either YEplac181GLexAE2-300B (Library C isolates) or YEplac181GLEXAE2N-300B (library A and B isolates) which contains an *Nhe*I site. Mutant LexA fusions were confirmed by gain (for library A and B isolates) or loss (library C) of the *Nhe*I site. The LexA fusion of the human chaperonin 10 cDNA was transferred from pEG202 to YEplac181GLexA202 (Chen *et al.,* 1994). Plate assays for transcriptional activation were performed as described above for full-length E2 on galactose/X-gal plates. All deletion mutants of BPV-1 E2 were constructed in the YEplac112GE2 vector. The VP16 transactivation domain (residues 410–490) was transferred to YEplac112GE2Δ54, YEplacc112GE2Δ139, and YEplac112GE2TR as a *Bam*HI fragment.

The transactivation potential of defective mutants was further characterized by quantitative β-galactosidase assay (Guarente and Mason, 1983). Selected mutants were transferred to the expression vector pBG331E2TR (Barsoum *et al.,* 1992) as *Bam*HI–*Kpn*I fragments generating a full-length E2 with mutation. Mutants were assayed for transcriptional activity in COS cells as previously reported (Grossel *et al.,* 1996). Total cell extracts from the electroporated COS cells lysed in denaturation buffer were analyzed for E2 proteins by immunoblot.

RESULTS

The transcriptional activation domain (TAD) of BPV-1 E2 has been previously described to encompass amino acids 1–220 (McBride *et al.,* 1989). Prior to undertaking an extensive screen for point mutations in this domain, we attempted to precisely delineate the limits of the E2 TAD in the yeast model. Deletion of the 15 amino-terminal residues of E2, with initiation at methionine 16, reduced transcriptional activation to background of the empty vector (Fig. 1A), as previously described (McBride *et al.,*

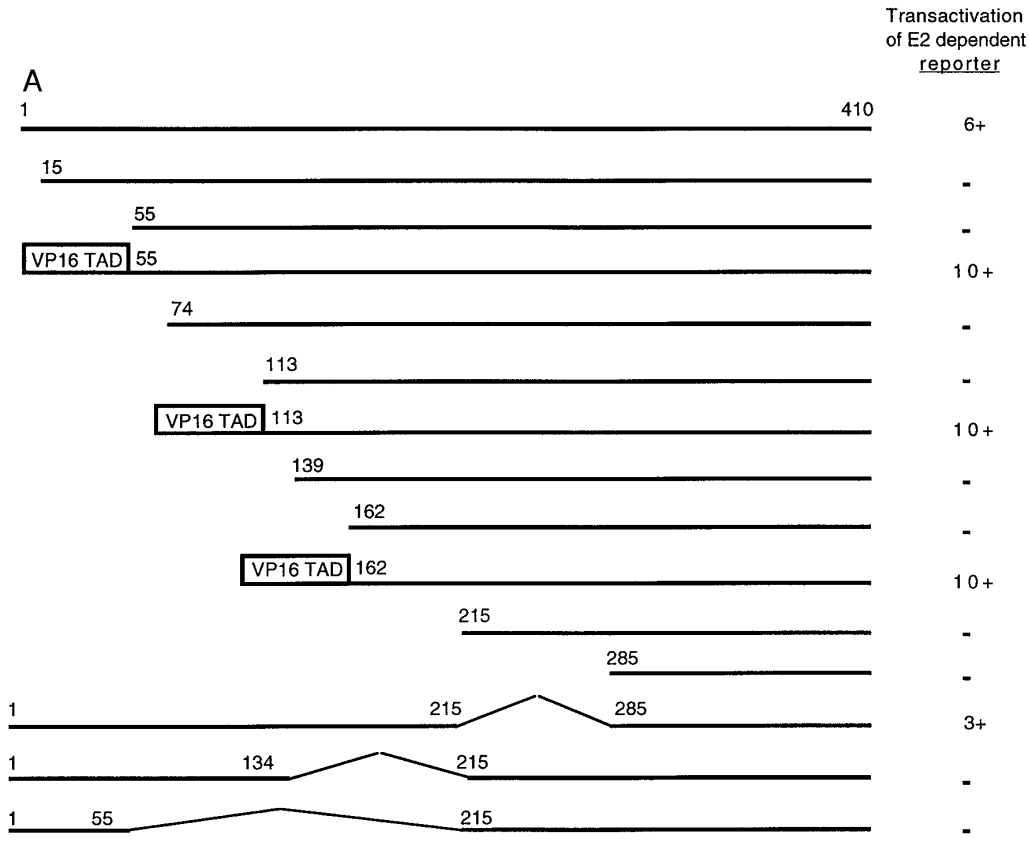

FIG. 1. (A) Transcriptional activation by BPV E2 deletion mutants in *S. cerevisiae.* E2 deletion mutants in YEplac112G were introduced in strain DBY1 along with E2 responsive LacZ reporter pBsy72. Four colonies for each mutant were transferred to galactose/X-gal minus tryptophan and uracil plates to induce E2 synthesis, and colony colors were scored at 24 and 48 hr (− indicates white; + indicates intensity of blue color). (B) Induction of LacZ activity of the LexA responsive promoter by LexA DNA binding domain fusions to regions of BPV-1 E2. The LexA–E2 chimeras were introduced into DBY1 along with the LexA responsive LacZ reporter pSH18-34. Four colonies for each construct were transferred to galactose/X-gal minus leucine and uracil plates and their color were scored at 24 and 48 hr (− indicates white; + indicates intensity of blue color).

1989). Truncated E2 proteins lacking amino acids 1–54 (Δ54), 1–74 (Δ74), 1–113 (Δ113), and 1–139 (Δ139) were completely defective for transcriptional activation. Fusion of the VP16 TAD (residues 410–490) to the E2Δ54 and E2Δ113 deletions resulted in potent activators which strongly induced the E2-dependent reporter as efficiently as VP16:E2TR (residues 162–410) (Lamberti *et al.,* 1990). These results indicate that the transcription defect of these N-terminal deletion mutants is a result of inactivation of the E2 TAD and imply that the C-terminal DNA binding domain functions were not abrogated by these perturbations. Internal in-frame deletions of amino acids 55–215 and 139–215 also completely abolished transcriptional activation. However, the 215–286 deletion, which removes the "hinge region" of E2, was able to activate transcription, although not as efficiently as WT E2, as has been previously reported (Winokur and McBride, 1992). Together, the phenotype of these mutants suggests that the BPV-1 E2 TAD comprises amino acids 1–215.

It has been recently reported that the E2 C-terminus including amino acids 310–410 binds the Pol II transcription factors TBP and TFIIB (Rank and Lambert, 1995). The functional significance of the interactions between TBP and TFIIB with the E2 DNA binding/dimerization domain remains to be determined. We have also detected binding of the E2 C-terminus to these basal factors (J.-M. Yao, D. Breiding, and E. Androphy, unpublished data). Mutations in this region of E2 that are transcriptionally defective in mammalian cells and retain dimerization and site-specific DNA binding have been described (Grossel *et al.,* 1996). To determine if the E2 transcriptional activation domain is able to function independently of the C-terminal DNA binding domain, the LexA DNA binding domain was fused to a similar set of E2 TAD deletions (Fig. 1B). Fusions containing the N-terminal 300, 284, and 216 residues activated the LexA-dependent LacZ reporter, while yeast expressing LexA202 and a fusion of LexA to human chaperonin 10 (LexA–SD22) remained white (data not shown). LexA–E2 1–54, LexA–E2 1–91, LexA–E2 1–113, LexA–E2 1–133, and LexA–E2 1–162 include

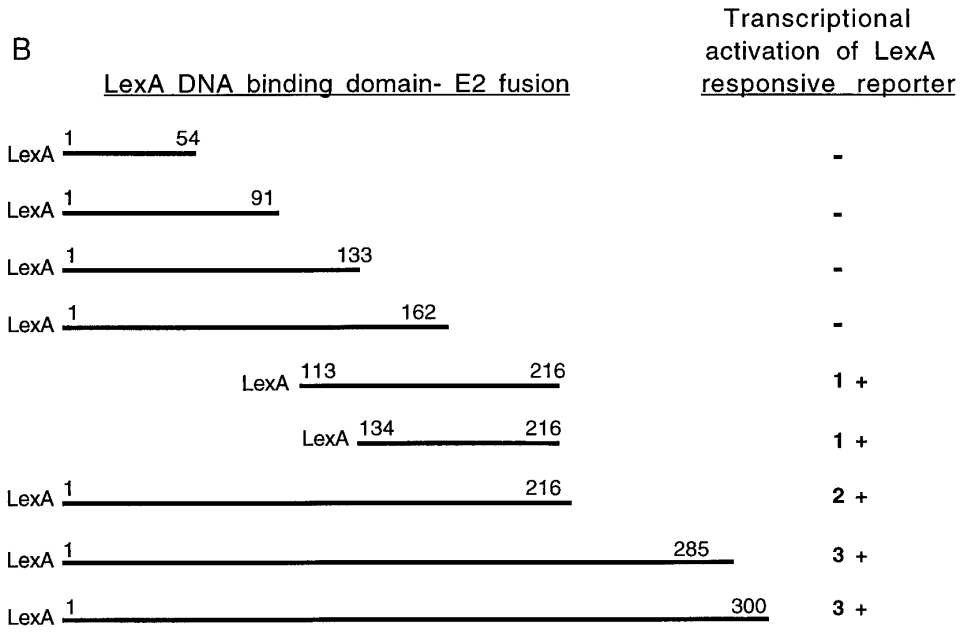

FIG. 1—*Continued*

the acidic regions of the E2 TAD and showed no transcriptional activity in this assay. Surprisingly, LexA–E2 113–216 and LexA–E2 133–216 weakly stimulated the LexA reporter. These LexA fusions possessed two- to threefold less activity than LexA–E2 1–216, indicating the requirement for amino-terminal residues for full activity.

Taken together these experiments demonstrate that E2 TAD is able to function independently of the C-terminus of E2 in yeast and that full activity of this domain requires residues 1 to 215. To identify amino acids in this domain necessary for transcriptional activation, we used a random mutagenesis scheme followed by phenotypic selection in yeast to isolate defective point mutants. Three libraries spanning residues 1–71, 71–134, and 134–215, designated A, B, and C, respectively, were generated by polymerase chain misincorporation mutagenesis (Leung *et al.,* 1988). These were introduced into the context of full-length BPV-1 E2 in the yeast expression vector YEplac112GE2. The libraries were transformed into DBY1 carrying an E2-dependent LacZ reporter pBsy72. Colonies were screened by replica plating to media containing galactose to induce E2 synthesis. Total cell protein extracts from white and light blue colonies were examined for the presence of full-length E2 by immunoblot to eliminate mutations which introduce termination codons or frameshifts. Plasmids carrying the E2 mutation were isolated, transformed into fresh yeast, and tested on galactose/X-gal plates. Those which retained the defective phenotype were further characterized by quantitative β-galactosidase assay and DNA sequence analysis. Through this phenotypic screen it was possible to identify mutants which have a 50% or greater reduction in β-galactosidase activity. In some cases mutants were obtained by direct sequence analysis of individual colonies from the bacterial plasmid library. These isolates were also subjected to immunoblot analysis to confirm the presence of E2. As expected, in addition to point mutations, each misincorporation library yielded at least 40 isolates with two or more mutations. Characterization of mutants obtained by phenotypic screening, when compared with those identified from library A by direct sequence analysis, indicated that this screen identified substitutions at residues critical for transcriptional activation from a random pool of mutations. Seven single-hit mutations identified by direct sequence analysis spanned residues 23 and 62, and with the exception of L54P (14% WT E2) all retain at least 40% or more of WT activity (Table 1, Figure 2). Seven highly defective point mutants (less than 2% of WT activity) were isolated in the phenotypic screen and six fall between residues 14 and 40 (Table 1). The seventh, Q66R, is near a cluster of highly defective mutations obtained from library B (residues 71–134). Mutants from library B are highly defective, and substitutions fall between amino acids 86 and 107. Screening of library C yielded six transactivation defective mutants of which three, Y138H, W145R, and R208G, possessed 8% or less of wild-type E2 activity.

Point mutants which exhibit a severe transcriptional activation defect were chosen from each library and assayed for transcriptional activation in *S. cerevisiae* as fusions of the LexA DNA binding domain to E2 residues 1–300. Since this fusion lacks the C-terminal E2 DNA binding and dimerization domain, it might distinguish mu-

TABLE 1

Transcriptional Activation by BPV-1 E2 Mutants in *S. cerevisiae*

	Mutant	% wild-type activity	
Library A residues 1–71	Q 15 H	<1	
	S 23 G	1	
	D 24 A[a]	75	
	K 25 E[a]	76	
	H 29 R	1	
	I 30 M[a]	42	A1 amino acids 1–40; net negative charge −3
	L 31 P	<1	
	L 31 Q	18	
	Y 32 H	<1	
	E 39 G	<1	
	A 46 E[a]	88	
	V 53 A[a]	82	
	L 54 P[a]	14	
	C 57 R	23	
	H 61 R[a]	53	
	Q 66 R	<1	
Library B residues 71–133	F 87 S	<1	A2 amino acids 65–90; net negative charge −2
	W 92 R	<1	
	S 93 P	<1	
	W 99 C	<1	
	E 105 G	13	
	P 106 S	<1	
	N127 Y	14	
	W 130 R	16	
Library C residues 134–216	Y 138 F	23	
	Y 138 H	<1	
	W 145 R	<1	
	E 176 G	23	
	R 179 G	17	
	S 181 F	16	
	R 208 G	8	

[a] Mutants identified by direct DNA sequence analysis of isolates from misincorporation library.

tations within the E2 N-terminus which disrupt function of the C-terminal E2 domain. All E2 mutants which were highly defective for transactivation (less than 2% WT E2) were similarly defective when assayed as LexA fusions (Table 2). One mutant (N127Y, 14% of WT E2) appeared to transactivate as well or better than wild type when tested as a LexA fusion. These results suggest that with the exception of N127Y, all mutant phenotypes can be attributed to specific inactivation of the E2 transcriptional activation domain.

Selected mutants from each misincorporation library which are highly defective in yeast were further characterized for transcriptional activation in COS-7 cells (Table 3). The transcriptional activation phenotype of these mutants in mammalian cells parallels the results in yeast and indicates that the yeast phenotypic screen identified residues critical to E2 function. Highly defective mutants from libraries A (Q15H, E39G), B (F87S, W92R, and W99C), and C (Y138H, W145R) were found to be similarly defective in COS cells and yeast. This suggests that the three clusters of defective mutants which span residues 15–39, 66–106, and 138–145 represent interdependent functional domains required for transcriptional activation in both yeast and mammalian cells. Western blots showed that these mutant proteins were expressed, although some, including D24A, K25E, F87S, W99C, and W145R, were at levels less that WT E2 (Fig. 3). However, this reduction in protein level does not account for their transcriptional defect. For example, while the amount of D24A protein present was very low, it demonstrated 36% of WT activity. In contrast, mutant proteins such as F87S, W99C, and W145R were expressed at higher levels than D24A yet were highly defective for transcriptional activation.

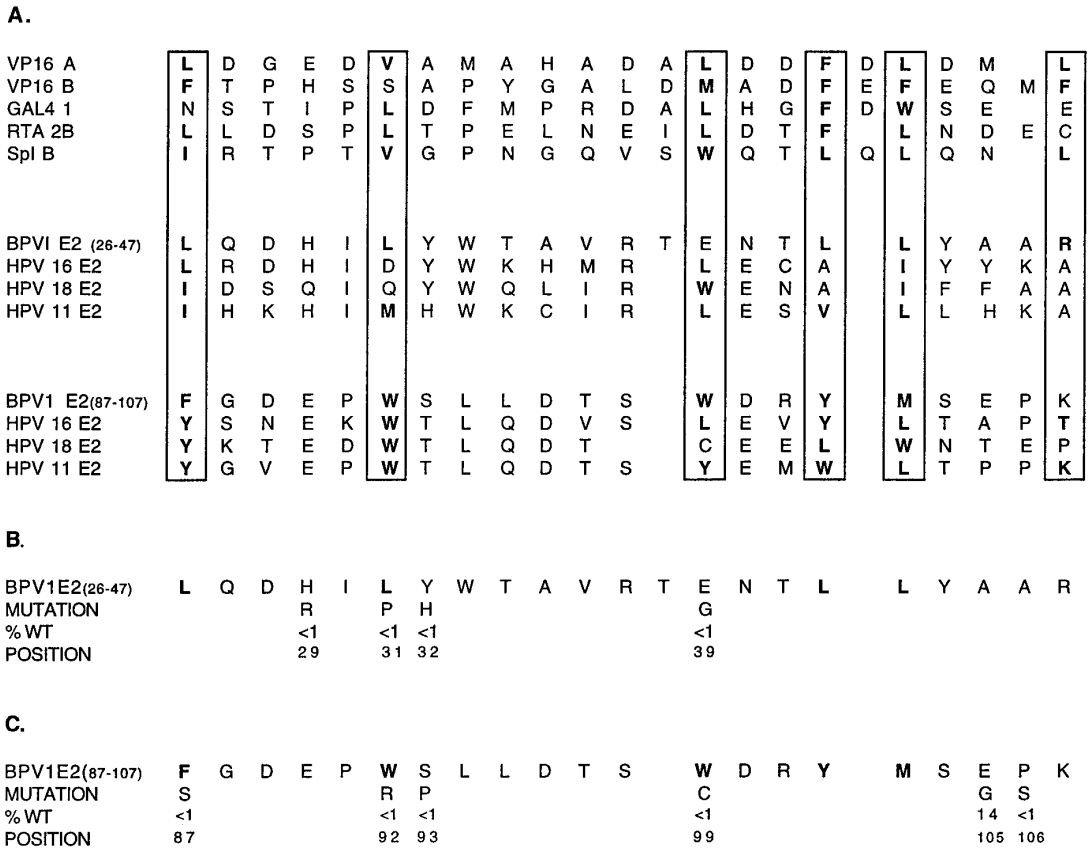

FIG. 2. Alignment of bulky hydrophobic residues in the E2 and other transcriptional activation domains. (A) Alignment of bulky hydrophobic residues in transactivation domains of VP16, GAL4, RTA, and Sp1 (Cress and Triezenberg, 1991; Hardwick *et al.,* 1992; Regier *et al.,* 1993; Blair *et al.,* 1994; Gill *et al.,* 1994) with motifs in BPV-1 and HPV-11, -16, and -18 E2. (B) Transactivation defective mutations in this interval of BPV-1 E2 are shown along with the bulky hydrophobic motif. Below each amino acid substitution the transactivation phenotype in *S. cerevisiae* is listed as percentage of wild-type BPV-1 E2 activity. (C) Same as for B, except that the motif and mutations span the 87 to 106 interval of BPV-1 E2.

DISCUSSION

Early studies have shown that the amino-terminal 215 amino acids of the BPV-1 E2 protein are necessary for transcriptional activation (McBride *et al.,* 1989); however, little is known of the structural and functional organization of the E2 transcriptional activation domain. While this article was in preparation, point mutations were reported which examine the role of the BPV-1 and HPV-16 E2 TADs in viral DNA transcription and replication (Brokaw *et al.,* 1996; Sakai *et al.,* 1996). From this study it is clear that many conserved residues throughout the E2 TAD are necessary for transcriptional activation. We chose to isolate transactivation defective point mutations in this domain using a functional screen in *S. cerevisiae*, a model previously used to characterize the E2 C-terminal DNA binding and dimerization domain (Prakash *et al.,* 1992). Libraries of random mutations in the E2 gene were screened for inability to activate an E2-dependent promoter. It is probable that these E2 mutants will include proteins that would be misfolded, unstable, or unable to bind cellular factors necessary for the E2 transcription or processing pathways.

Fourteen point mutations isolated by phenotypic screening demonstrated complete loss of E2 transcriptional activation. The fact that we could isolate single amino acid changes that were severely crippled for transcriptional activation was not predictable. Two lines of evidence suggest that most of the highly defective mutations affect a specific function of the E2 TAD. First, all 14 were totally defective for transactivation when assayed in the context of LexA DNA binding domain fusion to the E2 TAD (amino acids 1–300) into which the mutations were transferred (Table 2). Second, all mutant E2 proteins except W92R and P106S were able to interact with BPV-1 E1 in a two-hybrid assay, indicating that this E2 TAD function is preserved (N. Moscufo, D. Breiding, and E. Androphy, unpublished data). The phenotype of P106S may be caused by a nuclear localization defect, since another substitution of this residue has been reported to be defective for this function (Brokaw *et al.,* 1996; Skiadopoulos and McBride, 1996).

Early genetic analysis of the VP16 and GAL4 transaction domains indicated a correlation between overall net negative charge and transcriptional activity (Gill and

TABLE 2

Transactivation in *S. cerevisiae* by E2 TAD Mutants in LexA–E2 Fusions

Wild type	+++
Q 15 H	−
S 23 G	+/−
H 29 R	−
L 31 P	−
E 39 G	−
L 54 P	+
Q 66 R	−
F 87 S	−
W 92 R	−
W 99 C	−
E 105 G	+/−
P 106 S	−
N 127 Y	+++
W 130 R	−
Y 138 H	−
W 145 R	−
E 176 G	+
R 179 G	+
S 181 F	+
R 208 G	−

Note. E2 mutations were transferred to the galactose inducible LexA fusion vector YEplac181GLexAE2-300 and transformed into yeast strain DBY1 containing the LexA/LacZ reporter pSH18-34. For each mutant 4 colonies were innoculated on galactose/x-gal plates and scored for blue color formation at 24 and 48 hr (− indicates white and + indicates intensity of blue color).

Ptashne, 1987; Cress and Triezenberg, 1991). Substitution of individual acidic residues in these activation domains reduced transactivation, but did not result in a highly defective phenotype. Dramatic reductions in the activity of VP16 (10% or less of wild type) could only be achieved by combination of several mutations which reduced the net negative charge from −8 to −2 (Cress and Triezenberg, 1991). Activity of the cryptic N-terminal GAL4 activation domain I showed a similar dependence on charge, suggesting that a charge threshold may influence the function of acidic activation domains (Gill and Ptashne, 1987). Mutation of acidic residues in the C-terminal TAD of GAL4 (amino acids 854–874) revealed an imperfect correlation between negative charge and activation; however, uncharged or positively charged substitutions activated to near wild-type levels (Leuther *et al.,* 1993). Whether negatively charged amino acids are relevant for function of acidic activators has been questioned (Hahn, 1993).

The E2 transcriptional activation domain contains two regions of potential acidic amphipathic helical conformation (Giri and Yaniv, 1988) , A1 (amino acids 1–50) and A2 (amino acids 65–90). Residues 1–40 of A1 possess a net charge of −3 and A2 has a net charge −2. Deletion of either motif abolishes E2-stimulated transcription in yeast and mammalian cells (Giri and Yaniv, 1988; Lambert *et al.,* 1989; McBride *et al.,* 1989). However, unlike the VP16 TAD, these regions of E2 did not possess independent transcriptional activity when assayed in the context of LexA fusions in yeast, or with internal deletions which retain the E2 DNA binding domain (Fig. 1B) (Lambert *et al.,* 1989). Similar in-frame deletions which retain regions A1 and A2 were also unable to activate transcription in mammalian cells (Giri and Yaniv, 1988; Haugen *et al.,* 1988; McBride *et al.,* 1989).

Two clusters of highly defective point mutations isolated in this phenotypic screen fall in regions A1 and A2. Mutations which reduce the net negative charge of the A1 (Q15H, Y32H, and E39G) and A2 (Q66R) regions essentially abolished transcriptional activation in both yeast and mammalian cells. One exception, D24A, retained significant activity in yeast (75% of WT) and COS-7 cells (57% of WT). These results contrast with the effects of point mutations in the VP16 and GAL4 activation domains. These E2 mutations were less conservative than the engineered aspartic acid to asparagine substitutions used to analyze the role of charge in VP16 function, and this may contribute to the severity of some E2 mutant phenotypes. Radical changes at individual acidic positions of GAL4 activation domain I did not severely compromise function (Gill and Ptashne, 1987). It has been recently reported that E39D, which does not affect the charge of region A1, unlike E39G described in this study, is similarly defective for transactivation (Brokaw *et al.,* 1996). This suggests that the substitutions of specific amino acids, independent of charge, alter E2 function and could be responsible for the defective phenotypes of some mutants reported here. Furthermore, the phenotype of K25E, which increases the negative charge of amino acids 1–40 from −3 to −5, indicates that E2 transactivation is not directly proportional to its net charge.

TABLE 3

Transcriptional Activation by BPV1 E2 Mutants in COS-7 Cells

Construct	% wild-type activity
Wild type	100
Q 15 H	3
D 24 A	36
K 25 E	77
E 39 G	3
F 87 S	3
W 92 R	2
W 99 C	3
N 127 Y	44
Y 138 H	9
W 145 R	1

Note. Trans-activation by wild-type E2 and E2 mutants in COS-7 cells. Plasmid pBG331 carrying wt E2, mutant, or vector alone were electroporated into COS-7 cells with an E2-dependent growth hormone (hGH) reporter and media were assayed for hGH.

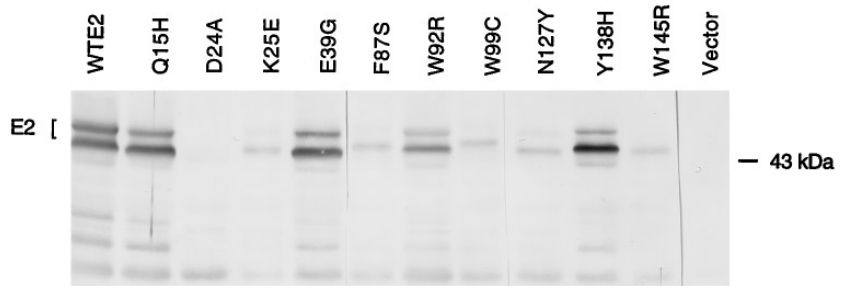

FIG. 3. Immunoblot of wild-type and E2 mutant proteins expressed in COS-7 cells.

Mutations which increased the acidity of the GAL4 activation domain I resulted in greater activity than wild type (Gill and Ptashne, 1987; Gill *et al.,* 1990), while E2 K25E had only 75% of wild-type activity in yeast and mammalian cells. In addition, our phenotypic screen has identified two transcriptionally defective mutations, S23G and L31P, which do not affect the charge of region A1.

Regions A1 and A2 of BPV-1 E2 were examined for the pattern of BH amino acids, as these have been shown to form important motifs in both acidic and positively charged transcriptional activators (Cress and Triezenberg, 1991; Hardwick *et al.,* 1992; Regier *et al.,* 1993; Gill *et al.,* 1994). As with the other activation domains, more than one alignment of BH residues can be made (Hardwick *et al.,* 1992). Figure 2A shows the alignment maximizing the number of BH residues at consensus positions. Four BH residues in the region A1 motif (residues 26 to 49) fall at consensus positions. However, only one mutation affecting a BH consensus position, L31P, was identified by phenotypic screening and showed less than 1% of WT activity in yeast and COS cells (Fig. 2C). A second substitution at this residue, L31Q isolated by direct sequence analysis of library isolates, also displayed reduced activity in yeast (18% of WT). The lack of correlation between mutations which inactivate transcriptional activation and BH consensus positions in region A1 of E2 suggests that this motif is not critical. In this respect the BH motif within region A1 could be similar to a subdomain of the VP16 TAD. Mutations at BH consensus positions in the VP16 B subdomain (residues 457–490) do not severely affect transcriptional activation (Regier *et al.,* 1993). In contrast, function of the VP16 A subdomain (residues 410–456) is highly sensitive to nonconservative substitutions at BH consensus positions (Regier *et al.,* 1993).

A second BH motif which spans E2 residues 87–107 appears to be analogous to VP16 A (Fig. 2A). Highly defective point mutations affecting three of four BH consensus positions (F87S, W92R, and W99C) were identified in the phenotypic screen (Fig. 2C). Of six mutations isolated in this region of E2, three affected BH consensus positions. The other three highly defective mutations involved radical substitutions within the BH consensus motif (S93P, E105G, and P106S). This correlation implies that the 87–107 interval of E2 defines a functional activation unit and is thus similar to the VP16 A and RTA domains. As with BH consensus positions in VP16 A (Regier *et al.,* 1993), transactivation by E2 is only partially impaired by substitution of tryptophan 92 with another BH residue, phenylalanine (Brokaw *et al.,* 1996). Unlike VP16 A, this E2 motif does not lie entirely within a region of net negative charge, only partially overlapping with the A2 interval (residues 64 to 94). In addition, while mutational analysis shows that this motif is critical to E2 function, it does not activate transcription independently in yeast (Fig. 1). Differences between the VP16 A and B domains have been described at the level of protein–protein interaction: while VP16 B associates with the $TAF_{II}40$ component of TFIID *in vitro,* VP16 A does not (Goodrich *et al.,* 1993).

Our genetic studies indicate that a function essential to transcriptional activation by E2 in yeast and mammalian cells lies between residues 127 and 209 (Tables 1 and 2). Consistent with this analysis, E2 residues 134 to 216 possess the ability to activate transcription in yeast when fused to the LexA DNA binding domain, though less efficiently than a LexA fusion to E2 residues 1 to 216 (Fig. 1). Although this region of E2 does not appear to resemble any known transcriptional activation domain, some of the most defective mutants (W130R, Y138F/H, and W145R) affect bulky hydrophobic amino acids. It does not appear that W130 and W145 fall within BH motifs as described in Fig. 2A. In addition, W145 is highly conserved in other papillomavirus E2 proteins (Giri and Yaniv, 1988), but no evidence for a BH motif was found in this region of human papillomavirus E2 ORFs. Further evidence for the functional importance of the 134–216 interval of E2 comes from a previous genetic study which described a mutant of BPV-1 E2 (E2ts1) that is temperature sensitive for transcriptional activation and episomal growth in the context of the BPV-1 genome in mouse C127 cells (DiMaio and Settleman, 1988). This mutation was constructed by insertion of Pro–Arg–Pro–Arg between S181 and T182. In the yeast phenotypic screen we identified S181F, which is partially defective for transcriptional activation (Table 1).

The large E2 TAD likely interacts with several cellular proteins to activate transcription. Our data suggest that it is composed of multiple interdependent domains. Relatively little is known about proteins which interact with the E2 transcriptional activation domain. It has been reported that the region of E2 spanning residues 161 to 282 enhances binding of TBP to the TATA sequence (Steger *et al.,* 1995). The VP16 TAD is known to physically interact with several cellular factors, including basal transcription factors (TBP, TFIIB), a TAF ($dTAF_{II}40$), and a coactivator (PC4, see discussion) (Ge and Roeder, 1994). The E2 mutants described in this study should prove useful to decipher the physiological relevance of what could prove to be multiple E2 protein–protein interactions.

ACKNOWLEDGMENTS

We thank James Richards and Meara Androphy for expert technical assistance. This work was supported by NIH Grant R01 CA58376. M.J.G. was supported by NIH T32 A107422-10.

REFERENCES

Alani, E., Cao, L., and Kleckner, N. (1987). A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics* 116, 541–545.

Androphy, E. J., Lowy, D. R., and Schiller, J. T. (1987a). Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. *Nature* 325, 70–73.

Androphy, E. J., Schiller, J. T., and Lowy, D. R. (1987b). Analysis of the sequence-specific DNA-binding activity of the bovine papillomavirus E2 trans-activating protein. *Cancer Cells/Papillomaviruses* 5, 79–85.

Ausbel, M. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds. (1993). "Current Protocols in Molecular Biology." Greene Publishing Associates, Boston.

Barsoum, J., Prakash, S. S., Han, P., and Androphy, E. J. (1992). Mechanism of action of the papillomavirus-E2 repressor — Repression in the absence of DNA binding. *J. Virol.* 66, 3941–3945.

Blair, W. S., Bogerd, H. P., Mador, S. J., and Cullen, B. R. (1994). Mutational analysis of the transcriptional activation domain of Rel A: Identification of a highly synergistic minimal acid activation module. *Mol. Cell. Biol.* 14, 7226–7231.

Brokaw, J. L., Blanco, M., and McBride, A. A. (1996). Amino acids critical for the functions of the bovine papillomavirus type 1 E2 transactivator. *J. Virol.* 70, 23–29.

Chen, J., McNealy, D., Dalal, D., and Andophy, E. J. (1994). Isolation, sequence analysis and characterization of a cDNA encoding human chaperonin 10. *Biochim. Biophys. Acta* 1219, 189–190.

Cress, W. D., and Triezenberg, S. J. (1991). Critical structural elements of the VP16 transcriptional activation domain. *Science* 251, 87–90.

DiMaio, D., and Settleman, J. (1988). Bovine papillomavirus mutant temperature sensitive for transformation, replication and transactivation. *EMBO J.* 7, 1197–1204.

Dostatni, N., Thierry, F., and Yaniv, M. (1988). A dimer of BPV-1 E2 containing a protease resistant core interacts with its DNA target. *EMBO J.* 7, 3807–3816.

Ge, H., and Roeder, R. G. (1994). Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of Class II genes. *Cell* 79, 513–523.

Gietz, R. D., and Sugino, A. (1988). New yeast–E. coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six base pair restriction sites. *Gene* 74, 527–534.

Gill, G., Pascal, E., Tseng, Z. H., and Tjian, R. (1994). A glutamine-rich hydrophobic patch in transcriptional factor Sp1 contacts the dTAFII110 component of drosophila TFIID complex and mediates transcriptional activation. *Proc. Natl. Acad. Sci. USA* 91, 1891–1896.

Gill, G., and Ptashne, M. (1987). Mutants of Gal4 protein altered in an activation function. *Cell* 51, 121–126.

Gill, G., Sadowski, I., and Ptashne, M. (1990). Mutations that increase the activity of a transcriptional activator in yeast and mammalian cells. *Proc. Natl. Acad. Sci. USA* 87, 2127–2131.

Giri, I., and Yaniv, M. (1988). Structural and mutational analysis of E2 trans-activating proteins of papillomaviruses reveals three distinct functional domains. *EMBO J.* 7, 2823–2829.

Goodrich, J. A., Hoey, T., Thut, C. J., Admon, A., and Tjian, R. (1993). Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIID. *Cell* 75, 519–530.

Grossel, M. G., Prakash, S. S., Barsoum, J., and Androphy, E. J. (1996). The BPV-1 E2 DNA contact helix cysteine is required for transcriptional activation but not replication in mammalian cells. *Virology* 217, 301–310.

Guarente, L., and Mason, T. (1983). Heme regulates transcription of the CYC1 gene of S. cerevisiae via an upstream activation site. *Cell* 32, 1279–1286.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* 75, 791–803.

Hahn, S. (1993). Structure (?) and function of acidic transcription activators. *Cell* 72, 481–483.

Ham, J., Dostatni, N., Arnos, F., and Yaniv, M. (1991). Several different upstream promoter elements can potentiate transactivation by the BPV-1 E2 protein. *EMBO J.* 10, 2931–2940.

Hardwick, J. M., Tse, L., Applegren, N., Nicholas, J., and Veliuona, M. A. (1992). The Epstein–Barr virus R transactivator (RTA) contains a complex, potent activation domain with properties different from those of VP16. *J. Virol.* 66, 5500–5508.

Harrison, S. M., Gearing, K. L., Kim, S. Y., Kingsman, A. J., and Kingsman, S. M. (1987). Multiple cis-active elements in the long control region of bovine papillomavirus type 1. *Nucleic Acids Res.* 15, 10267–10284.

Haugen, T. H., Cripe, T. P., Ginder, G. D., Karin, M., and Turek, L. P. (1987). Trans-activation of an upstream early gene promoter of bovine papilloma virus-1 by a product of the viral E2 gene. *EMBO J.* 6, 145–152.

Haugen, T. H., Turek, L. P., Mercurio, F. M., Cripe, T. P., Olson, B. J., Anderson, R. D., Seidl, D., Karin, M., and Schiller, J. (1988). Sequence-specific and general transcriptional activation by the bovine papillomavirus-1 E2 trans-activator require an N-terminal amphipathic helix-containing E2 domain. *EMBO J.* 7, 4245–4253.

Hawley-Nelson, P., Androphy, E. J., Lowy, D. R., and Schiller, J. T. (1988). The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. *EMBO J.* 7, 525–531.

Lambert, P. F., Dostatni, N., McBride, A. A., Yaniv, M., Howley, P. M., and Arcangioli, B. (1989). Functional analysis of the papilloma virus E2 trans-activator in Saccharomyces cerevisiae. *Genes Dev.* 3, 38–48.

Lamberti, C., Morrissey, L. C., Grossman, S. R., and Androphy, E. J. (1990). Transcriptional activation by the papillomavirus E6 zinc finger oncoprotein. *EMBO J.* 9, 1907–1913.

Leung, D. W., Chen, E., and Goeddel, D. V. (1988). A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* 5, 11–18.

Leuther, K. K., Salmeron, J. M., and Johnston, S. A. (1993). Genetic evidence that an activation domain of GAL4 does not require acidity and may form a β sheet. *Cell* 72, 575–585.

Li, R., Knight, J. D., Jackson, S. P., Tjian, R., and Botchan, M. R. (1991). Direct interaction between Sp1 and the BPV enhancer E2 protein mediates synergistic activation of transcription. *Cell* 65, 493–505.

McBride, A. A., Byrne, J. C., and Howley, P. M. (1989). E2 polypeptides

encoded by bovine papillomavirus type 1 form dimers through the common carboxyl-terminal domain: Transactivation is mediated by the conserved amino-terminal domain. *Proc. Natl. Acad. Sci. USA* 86, 510–514.

McBride, A. A., Schlegel, R., and Howley, P. M. (1988). The carboxy-terminal domain shared by the bovine papillomavirus E2 transactivator and repressor proteins contains a specific DNA binding activity. *EMBO J.* 7, 533–539.

Morrissey, L. C., Barsoum, J., and Androphy, E. J. (1989). Trans-activation by the bovine papillomavirus E2 protein in Saccharomyces cerevisiae. *J. Virol.* 63, 4422–4425.

Moskaluk, C., and Bastia, D. (1988). Interaction of the bovine papillomavirus type 1 E2 transcriptional control protein with the viral enhancer: purification of the DNA-binding domain and analysis of its contact points with DNA. *J. Virol.* 62, 1925–1931.

Moskaluk, C. A., and Bastia, D. (1989). The bovine papillomavirus type 1 transcriptional activator E2 protein binds to its DNA recognition sequence as a dimer. *Virology* 169, 236–238.

Prakash, S. S., Grossman, S. R., Pepinsky, R. B., Laimins, L. A., and Androphy, E. J. (1992). Amino acids necessary for DNA contact and dimerization imply novel motifs in the papillomavirus E2 trans-activator. *Genes Dev.* 6, 105–116.

Rank, N. M., and Lambert, P. F. (1995). Bovine papillomavirus type 1 E2 transcriptional regulators directly bind two cellular transcription factors, TFIID and TFIIB. *J. Virol.* 69, 6323–6334.

Regier, J. L., Shen, F., and Triezenberg, S. J. (1993). Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. *Proc. Natl. Acad. Sci. USA* 99, 883–887.

Sakai, H., Yasugi, T., Benson, J. D., Dowhanick, J. J., and Howley, P. M. (1996). Targeted mutagenesis of the human papillomavirus type 16 E2 transactivation domain reveals separable transcriptional activation and DNA replication functions. *J. Virol.* 70, 1602–1611.

Skiadopoulos, M. H., and McBride, A. A. (1996). The bovine papillomavirus type 1 E2 transactivator and repressor proteins use different nuclear localization signals. *J. Virol.* 70, 1117–1124.

Spalholz, B. A., Lambert, P. F., Yee, C. L., and Howley, P. M. (1987). Bovine papillomavirus transcriptional regulation: localization of the E2-responsive elements of the long control region. *J. Virol.* 61, 2128–2137.

Stanway, C. A., Sowden, M. P., Wilson, L. E., Kingsman, A. J., and Kingsman, S. M. (1989). Efficient activation of transcription in yeast by the BPV1 E2 protein. *Nucleic Acids Res.* 17, 2187–2196.

Steger, Ham, J., Lefebrve, O., and Yaniv, M. (1995). The bovine papillomavirus E2 protein contains two activation domains: One that interacts with TBP and another that functions after TBP binding. *EMBO J.* 14, 329–340.

Ushikai, M., Lace, M. J., Yamakawa, Y., Kono, M., Anson, J., Ishiji, T., Parkkinen, S., Wicker, N., Valentine, M. E., Davidson, I., Turek, L. P., and Haugen, T. H. (1994). Trans-activation by the full-length E2 proteins of human papillomavirus type 16 and bovine papillomavirus type 1 in vitro and in vivo: Cooperation with activation domains of cellular transcription factors. *J. Virol.* 68, 6655–6666.

Winokur, P., and McBride, A. (1992). Separation of the transcriptional activation and replication functions of the bovine papillomavirus-1 E2 protein. *EMBO J.* 11, 4111–4118.