The EMBO Journal vol.9 no.6 pp.1907 – 1913, 1990

Transcriptional activation by the papillomavirus E6 zinc finger oncoprotein

Carmela Lamberti[1], Lloyd C.Morrissey[1], Steven R.Grossman[2] and Elliot J.Androphy[1,3]

[1]Department of Dermatology, New England Medical Center and Tufts University School of Medicine, Boston, MA 02111, [2]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637 and [3]Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111, USA

Communicated by F.Cuzin

The introduction of the bovine (BPV) or human papillomavirus E6 gene into susceptible cells can result in their transformation, but there are few clues to the mechanism of action of the E6 gene. The characteristic features of E6 proteins are their small size (~150 amino acids) and the potential to form two large zinc fingers. To determine if E6 can function as a transcription factor, the BPV E6 gene was fused to the sequence specific DNA binding peptide encoded by the BPV E2 gene. This chimeric E6–E2 protein *trans*-activated promoters that incorporated E2 binding elements in both rodent cells and *Saccharomyces cerevisiae*. In the absence of E6–E2 localization to the target promoter, *trans*-activation did not occur. Alteration of the cysteine residues at the base of each finger abrogated the transcriptional activity of the E6–E2 hybrids. These data demonstrated that the BPV E6 gene encodes a transcription activation domain and imply that a specific structure of the protein, most likely the zinc fingers, is critical for this function. Since these cysteine mutants are also transformation defective, E6 transcriptional functions may be required for its oncogenic activity.

Key words: E6 proteins/papillomavirus/transcription/transformation/zinc finger

Introduction

Human (HPV) and animal papillomaviruses have been implicated in the etiology of benign and malignant epithelial tumors. The 137 amino acid bovine papillomavirus (BPV) E6 protein induced morphologic transformation of rodent cells *in vitro* (Schiller *et al.*, 1984; Androphy *et al.*, 1985). HPV E6 genes were less efficient in the focus formation assay than their bovine counterpart; however, several *in vitro* models have confirmed the role of HPV E6 genes in transformation (Bedell *et al.*, 1987; Iftner *et al.*, 1988; Cerni *et al.*, 1989; Hawley-Nelson *et al.*, 1989; Munger *et al.*, 1989; Watanabe *et al.*, 1989; Hudson *et al.*, 1990). Comparison of BPV E6 with human HPV E6 proteins reveals only moderate conservation of amino acids, most evident in the two pairs of cysteine repeats (Cys-x-x-Cys), postulated to mediate metal binding and the formation of a 'finger' conformation, and residues within the fingers (Cole and Danos, 1987). The E6 zinc fingers are unusually large, with 29–30 residues, and the two 'fingers' are separated by ~40 amino acids that share little homology among the PV E6 proteins. Mutational analysis of PBV E6 has verified the requirement of the cysteine motifs for cellular transformation (Vousden *et al.*, 1989). BPV and HPV E6 proteins have been localized by cell fractionation in part to nuclear matrix (Androphy *et al.*, 1985, 1987a; Grossman *et al.*, 1988), reported to bind DNA non-specifically (Mallon *et al.*, 1987) and shown to chelate zinc *in vitro* (Barbosa *et al.*, 1989; Grossman and Laimins, 1989), features common to several recently described transcription factors.

While these physical and functional observations suggest that E6 may operate as a transcriptional regulator, it is believed that co-ordination of PV gene expression is primarily performed by the viral E2 protein. Through its carboxy-terminal 100 amino acids, E2 binds a specific DNA sequence, shared among all PVs, which acts as an E2 protein dependent enhancer (Androphy *et al.*, 1987b; Hawley-Nelson *et al.*, 1988; McBride *et al.*, 1988). The amino-terminal 220 residues of E2 are required for this activation (Giri and Yaniv, 1988; Haugen *et al.*, 1988; McBride *et al.*, 1989). While the E2 transcriptional activation region (Giri and Yaniv, 1988; Haugen *et al.*, 1988) structurally resembles the acidic amphipathic helix found in a variety of transcription factors (Ptashne, 1988), E6 is a basic protein (pI ~ 10–11) and does not appear to form similar negatively charged motifs, nor is it similar to the glutamine rich region of transcription factor Sp1 (Courey and Tjian, 1988) or the proline repeats of NF-1 (Mermod *et al.*, 1989). A truncated form of E2 (called E2-R here) lacking a portion of the enhancer activating domain functionally repressed the E2 dependent enhancer (Lambert *et al.*, 1987).

The cloning and characterization of mammalian, yeast and viral transcription factors have revealed that these often encode discrete operational domains (Dynan, 1989; Mitchell and Tjian, 1989), and this feature allows the transfer of functional units to other proteins, or domain swapping. To test the hypothesis that E6 possesses transcriptional potential, we molecularly cloned the E6 gene onto the amino-terminus of the BPV E2 DNA binding domain, in effect substituting the E6 zinc finger protein for the E2 enhancer activation region. Our rationale for creating this chimeric gene was that, while an E6 responsive element was suggested to be present in the regulatory region of HPV 18 (Gius *et al.*, 1989), significant responses have not been reported with BPV or other HPVs (Spalholz *et al.*, 1987; Haugen *et al.*, 1988). The degree of transcriptional activation in transient assays is very low, in the range of 2- to 10-fold, suggesting a non-specific effect. Since BPV E6 can induce oncogenic changes without the contribution of any other viral sequences, this implied that if E6 functioned as a transcription factor, the requisite interactions for transformation are with cellular DNA or proteins and not viral sequences. Moreover, while the E6 gene may stimulate the viral upstream regulatory region (URR; or long control region), a DNA

element either directly bound by E6 or specifically activated by E6 has not been defined. The URR is a complex region that includes recognition sites for multiple cellular transcription factors as well as a replication origin, and hence in this context it is difficult to assign transcriptional functions directly to E6. Therefore, the junction of E6 to the E2 DNA binding domain permitted localization of E6 to minimal promoter units that included E2 binding sites as their upstream regulatory elements.

The transcriptional activity of the chimeric protein was examined in both mouse C127 and NIH 3T3 cells since BPV E6 morphologically transforms the former but not the latter. In both rodent cell lines and in *Saccharomyces cerevisiae*, transcription was enhanced upon localization of the E6–E2 hybrid to the promoter. Since transformation defective E6 mutations of the consensus cysteine motifs were unable to stimulate the target promoter, we speculate that the E6 zinc fingers may constitute a novel function for this structural motif as a transcriptional activator.

Results

Initially, we confirmed that the BPV E6 gene could not stimulate an SV40 based minimal promoter in rodent cells, even when the promoter included segments of the BPV regulatory region, as has been reported by other investigators (Spalholz *et al.*, 1987; Haugen *et al.*, 1987). Since a BPV E6 responsive DNA element has not been identified, the E6 coding region was molecularly cloned onto the DNA binding domain of BPV E2, creating E6–E2-R (Figure 1). This E2-R region lacked the E2 activation domain and is unable to induce E2 elements in mammalian cells and yeast (Lambert *et al.*, 1989; Morrissey *et al.*, 1989). The fusion gene was constructed to preserve the BPV E6 protein, with the only alteration of E6 limited to a conservative change of lysine to arginine two amino acids prior to its terminal proline. The carboxy-terminal four amino acids of E6 were otherwise intact, since their deletion had previously been shown to abrogate E6 transforming activity despite the sequence divergence of HPV and BPV E6 in this region (Vousden *et al.*, 1989).

When NIH 3T3 or C127 cells were co-transfected with the E6–E2-R chimera expressed from a retroviral long terminal repeat (LTR; Cepko *et al.*, 1984) along with a series of chloramphenicol acetyltransferase (CAT) reporter plasmids that contained permutations of the E2 binding sites (Hawley-Nelson *et al.*, 1988), expression of the CAT gene increased 30- to 50-fold above background levels (Table I). In comparison, the wild-type BPV E2 gene demonstrated activity similar to E6–E2-R with a single E2 responsive element. In contrast, while E2 produced a significant increase in activity with two and four E2 binding sites, E6–E2-R exhibited little variation. Quantities of CAT were consistently lower in C127 than NIH 3T3 cells; results for the latter are shown in Table I.

Two distinct controls indicated that the E6 moiety must be localized to the promoter region for this stimulation. First, a two amino acid insertion at nucleotide 3812 in the carboxy-terminus of E2 has been reported to inactivate the DNA binding domain of E2 (Haugen *et al.*, 1988), and after transfer of this mutation to the chimera as E6–E2-R-3812 (Figure 1), CAT production returned to basal levels (Table I). Second, E6–E2-R failed to enhance transcription

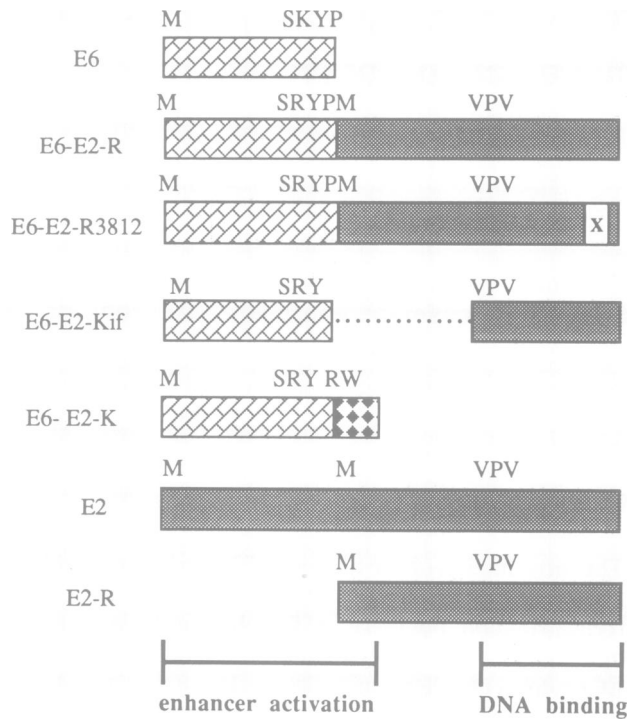

Fig. 1. Schematic diagram of constructions used in these experiments. ☑ = BPV E6 from first methionine (M) through terminal proline (P). In the E6–E2 chimeric proteins, the wild-type lysine (K) was replaced with an arginine (R) created within the oligonucleotides that fused E6 to E2. The E2 region ▨ in E6–E2-R begins with the methionine codon at nt 3089 in BPV, E6–E2-Kif deletes 122 amino acids from the E2 protein, coupling E6 to a smaller region of the E2 C-terminus. In E6–E2-K the E2 DNA binding domain is not in-frame with E6; the terminal E6 proline is lost and replaced with 22 amino acids ▩ (Y = tyrosine, W = tryptophan, S = serine, V = valine). The regions of E2 required for transcriptional activation and DNA binding are bracketed at the bottom of the figure.

Table I. Transactivation in NIH 3T3 cells[a]

Construction	0 E2 bs	1 E2 bs	2 E2 bs	3 E2 bs	2 mut E2 bs
pZipNeoSV(X)-1	1	1	1	1	1
E6	nd	1	1	1	1
E6–E2-R	2.6	52.5	27.5	47	2
E6–E2-R-3812	nd	1	1	1	1
E2	nd	37	92	168	1

[a]Transcriptional activity of E6, selected E6–E2 chimeras and E2 in calcium phosphate transfected mouse NIH 3T3 cells (Prakash *et al.*, 1989). The CAT reporters with 0–3 E2 binding sites (bs) are from Hawley-Nelson *et al.* (1988) and production of CAT was normalized to the control expression plasmid pZipNeoSV(X)-1 (Cepko *et al.*, 1987).

from a promoter with two mutated E2 binding sites as the upstream element. It has been previously reported that amino-terminally truncated E2 proteins such as E2-R do not activate the E2 enhancer motif in rodent (Lambert *et al.*, 1987; Haugen *et al.*, 1988) and yeast cells (Morrissey *et al.*, 1989).

Since PV infection and pathologic effects are generally limited to epithelial cells (an exception is fibroma in some animals) we questioned whether E6 *trans*-activation function was operative in the lower eukaryote *S.cerevisiae*. This model also afforded several advantages. Cell lines were

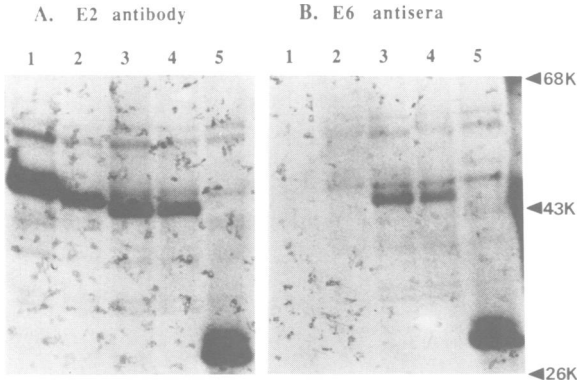

Fig. 2. Immunoprecipitation of yeast extracts with BPV E2 monoclonal antibody B202 (**A**) or with BPV E6 antisera (**B**). Lanes 1 = E2, lanes 2 = E2-793f, lanes 3 = E6−E2-R, lanes 4 = E6−E2-R-3812, lanes 5 = E6−E2-Kif.

isolated that maintained both the E6−E2 and reporter plasmids through linked selectable genes. Physiological effects of E6 were minimized since the yeast could be expanded in media supplemented with glucose, thereby repressing the GAL upstream activation sequence (UAS) that controlled PV protein expression. The ability of E2 binding site motifs to act as an E6−E2 dependent UAS when placed 5′ to a *lacZ* gene could be rapidly and quantitatively measured with a colorimetric assay for the enzyme β-galactosidase.

In addition, the fidelity of the protein coding regions of these E6 constructions could be confirmed by labeling yeast cells grown in galactose with [^{35}S]methionine or by immunoblot. The predicted 40 kd E6−E2-R protein was immunoprecipitated by both a BPV E2 specific monoclonal antibody (B202; E.Androphy, unpublished) and antisera directed against BPV E6 (Figure 2, lanes 3A and B). Similar results were obtained with E6−E2-R-3812 (lanes 4A and B) and the in-frame deletion of 122 amino acids from the E2-R segment of E6−E2-R (E6−E2-Kif in Figure 1 at 30 kd; lanes 5A and B in Figure 2). As expected, E2 and E2-793f, an in-frame deletion of 15 amino acids in the amino-terminus of E2 (Haugen *et al.*, 1988), were only precipitated with the E2 monoclonal, while the opposite occurred for the E6 protein expressed from the yeast vector. E6−E2-K (Figure 1) was precipitated by E6 specific antisera and migrated faster (~20 kd) since the E2 coding region is out-of-frame. Longer exposures of the immunoprecipitations and immunoblots confirmed expression of E6 and E6−E2-K, which did not label efficiently with methionine (data not shown). Since the epitope recognized by the monoclonal antibody B202 is in the distal E2 'hinge' region (E.Androphy, unpublished) immediately downstream from the *Kpn*I site (amino acid 285), these data confirmed that the E2 portion of the E6−E2 hybrid is expressed.

When E6 alone (pYE6) was introduced into yeast strain BGW1-7a along with the *lacZ* gene that carried one, two or four copies of the E2 binding site (pBY-1, 2 or 4, respectively; Morrissey *et al.*, 1989), no significant increase in the expression of β-gal over levels with the vector pKP-15 were detected with cells cultured in galactose (Table II). The series of E6−E2 DNA binding domain fusion constructions were then co-transformed along with the reported promoter plasmids and colonies that contained both constructions were

Table II. Production of β-gal from an E2 responsive promoter in *S.cerevisiae*[a]

Construction	pBY-1	pBY-2	pBY-4
pKP-15	2.9	2.8	2.6
E6	2.4	2.8	2.9
E6−E2-R	18	44	30
E6−E2-R-3812	2.7	2.0	3.2
E6−E2-Kif	10.5	19.0	12.4
E6−E2-K	2.5	2.2	2.8
E2	17	42	211
E2-R	2.0	3.0	1.8
E2-793f	2.7	2.6	2.0
VP 16-E2	710	1020	1470
AH-E2	6.0	24.5	124

[a]pBY-1, -2 and -4 contain the *cyc*-1 TATA elements with one, two or four E2 binding sites upstream respectively (Morrissey *et al.*, 1989). Cells were harvested after 16 h culture in minimal media supplemented with 2.5% galactose. The results represent the average of two to four experiments and data are expressed as β-gal units (optical density at 420 nm × 1000/optical density at 600 nm, corrected for time).

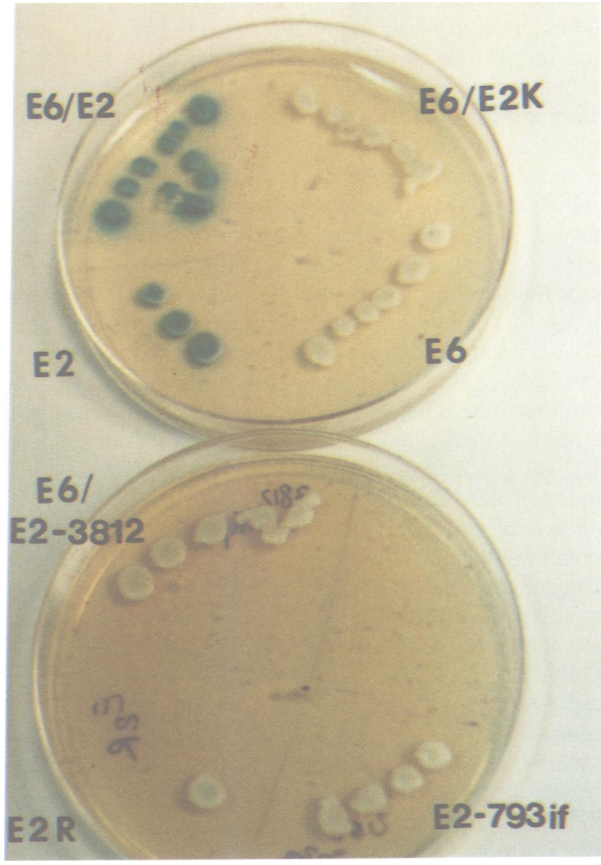

Fig. 3. Yeast transfected with E6, E2 or the E6−E2 hybrid proteins and the E2 binding site *lacZ* reporter plasmid pBY-4 (Morrissey *et al.*, 1989) were selected and transferred to minimal media plates with galactose as carbon source and the indicator substrate X-gal. E2 and E6−E2-R induced expression of β-gal staining the colonies blue.

selected on media deficient for uracil and leucine. In comparison to basal levels, β-gal expression increased ~10- to 20-fold with E6−E2-R (Table II). By culturing the cells on galactose containing plates supplemented with the *lacZ* substrate, X-gal, *trans*-activation by E2 and and E6−E2-R could be visualized. These yeast colonies were stained deep

Table III. The transactivation properties of point mutants in BPV E6 after transfer to the E2-R chimera were analyzed in *S.cerevisiae* with the E2 binding site *lacZ* reporter genes[a]

Construct	E6 mutation	β-Gal (units)			Transformation in E6 form
		pBY-1	pBY-2	pBY-4	
E6−E2-R	−	18	37	42	++++
E6-149−E2-R	C→S finger 1	4	4	4	−
E6-212−E2-R	I→T finger 1	9	20	31	+++
E6-368−E2-R	C→S finger 2	4	4	3	−
E6-460−E2-R	C→V finger 2	4	3	3	−

[a]The transforming ability of these E6 genes as previously reported (Vousden *et al.*, 1989) is summarized in the last column.
(C = cysteine, S = serine, I = isoleucine, T = threonine, G = glycine, V = valine.)

blue on indicator plates (Figure 3). The control constructions E2 793f and E2-R, which do not include the full E2 enhancer activation domain, failed to *trans*-activate the reporter series (Table II and Figure 3).

While one E2 binding element acted as an E6−E2-R dependent UAS, two E2 binding motifs demonstrated an additional 2- to 3-fold stimulatory effect. However, with a tandem of four sites, the expression of β-gal did not dramatically increase and in some experiments decreased. This contrasts with BPV E2, which yielded β-gal quantities similar to those of E6−E2 with one or two motifs, but increased β-gal expression an additional 5-fold with four E2 binding sites. Since the degree of activation with E6−E2-R and E2 is relatively low, a synthetic amphipathic helix (AH-E2; Giniger and Ptashne, 1987) and the strong activating region from the herpes virus VP-16 gene (VP16-E2; Sadowski *et al.*, 1988) were cloned onto an E2 DNA binding module. With one or two E2 binding sites, both E6−E2-R and E2 were twice as active as AH-E2, but with four E2 motifs, AH-E2 produced a 5-fold stimulation, as was observed with E2. VP16-E2 demonstrated high levels of β-gal production that were augmented with additional copies of the E2 UAS (Table II).

Since pYE6 failed to activate the minimal promoter containing the E2 UAS, this implied that the E6 required positioning near the transcription start site to exert its actions. As observed in rodent cells, E6−E2-R-3812 was defective for stimulation of the E2 motifs in *S.cerevisiae*. E6−E2-K, in which the E2 DNA binding domain is out of translational reading frame with E6, acted similarly. In E6−E2-Kif 122 amino acids of the E2 enhancer activating and 'hinge' domains present in E2-R were eliminated and the E2 DNA binding module was restored to the E6 reading frame. The E6−E2-Kif hybrid induced β-gal expression but levels were reproducibly ~40% lower than with E6−E2 (Table II).

It has been reported that random DNA segments from *Escherichia coli* can activate transcription in yeast when localized to a promoter (Ma and Ptashne, 1987). These activation regions are believed to form amphipathic helices with negatively charged residues on one surface. E6 does not appear to resemble these structures but instead probably exists in a zinc finger conformation. We therefore questioned whether the transcriptional activation reported here required the postulated metal fingers of the E6 protein, which could be disrupted by point mutation of the cysteine motifs (Vousden *et al.*, 1989). Three such E6 sequences E6-149, E6-368 and E6-460 were transferred to the yeast E6−E2-R chimeric vector. These three cysteine mutations (column 2 in Table III) affect the cysteine motif at the base of each putative finger. All were transformation defective in a focus formation and anchorage independent growth in semi-solid media. These same mutations in the construct E6−E2-R failed to activate the test promoter in *S.cerevisiae* (Table III). In contrast, a conservative mutation in the first finger, E6-212-E2-R was transcription competent. E6-212 has been reported to have wild-type transforming capability (Vousden *et al.*, 1989). These initial studies suggest that, in contrast to adenovirus type 5 E1a (Lillie *et al.*, 1987), BPV E6 transcription activation properties may be concordant with its transformation ability.

Discussion

We postulated that E6 may possess transcriptional properties like other nuclear transforming genes such as adenovirus E1a or the retroviral v-*jun*, v-*fos* and v-*myb* (Lillie *et al.*, 1987; Curran and Franza, 1988; Lech *et al.*, 1988). To test this hypothesis, a domain swap experiment was designed that exchanged the E2 enhancer activation region with BPV E6. This hybrid E6−E2 would recognize the E2 binding motifs ACCG N_4 CGGT (Androphy *et al.*, 1987b) in the test promoter through its E2 DNA binding region. The E6−E2 chimera was as efficient as E6 in focus formation assays (data not shown), indicating that the transcriptional properties attributed to E6 as a fusion protein did not alter its transforming potential. The synthesis of a spliced E6−E2 product in BPV transformed cells has not been reported, although this could be produced by an uncommon RNA.

The ability of the E6−E2-R construction to induce expression of a reporter gene was 20- to 50-times above background levels in two established mouse cell lines and in *S.cerevisiae*. Several control constructions provided support for the assignment of transcriptional activity to E6. Three point mutants of E6 failed to induce expression of the reporter gene when localized to the E2 dependent promoters. The in-frame deletion of fifteen amino acids from the amino-terminus of E2 (E2-793f) demonstrated that the synthesis of a similar length of peptide fused to the E2 DNA binding domain did not create an activator. E6−E2-R-3812, which differs from E6−E2-R by the insertion of two amino acids at the 3′ end of the E2 DNA binding domain, and the E6−E2-K construction, were *trans*-activation defective, providing strong evidence that the observations reported here are a direct outcome of E6 localization at the promoter. The E6−E2-R stimulation cannot be attributed to the residual portion of the E2 enhancer activation domain, since E6−E2-Kif was active, although its stimulatory effect was only one-third that with E6−E2. This may be a consequence of the loss of E2 region that itself is not sufficient but may contribute to transcription enhancement. The physical truncation of E6−E2-Kif could impair interactions with cellular transcription factors that ultimately lead to RNA polymerase activation. Alternatively, since the E6−E2-R chimera is larger than E6−E2-Kif (40 kd versus 16 kd respectively), E6−E2 may acquire an efficient nuclear localization signal that could be provided in its E2-R region. We therefore concluded that BPV E6 can substitute, in part, for the *trans*-activating domain of the BPV E2 protein,

defining a potential novel function for the BPV 1 E6 reading frame. This E6 encoded activity required targeting to the promoter for its detection.

The BPV E6−E2-R fusion protein stimulated transcription in yeast ~20- to 50-fold in several experiments. This is relatively low when compared with other viral factors such as E1a (Lillie *et al.*, 1989) or VP-16 (Sadowski *et al.*, 1988). However, with one or two E2 binding elements, the activity with E6−E2 was similar to the known PV enhancer activating gene E2. This level of stimulation was not caused by inefficient or inhibitory properties intrinsic to the E2-R DNA binding module, since VP-16-E2 strongly induced our reporter constructions. We hypothesize that E6 and E2 transcription properties reflect fundamental biological differences between the non-lytic papillomaviruses, which do not produce large quantities of viral mRNA in naturally occurring infection, and the lytic adeno and herpes viruses.

In all cells tested, the induction of the reporter by E6−E2 was maximal with two copies of the E2 DNA binding sites and did not significantly increase and in some cases decreased slightly with a tandem of four sites. In contrast, activation by E2 increased dramatically with additional copies of this motif. One explanation for this observation is that placement of several of the highly charged zinc containing E6 proteins in close apposition to each other is not energetically favorable. Alternatively close approximation of E6 molecules may hinder interactions with other proteins in the transcription cascade.

The artificial amphipathic helix-E2 and the activating region from the herpes virus VP16 gene-E2 demonstrated progressive augmentation of transcription with one, two and four E2 binding sites. Since E2 and VP-16 are believed to activate transcription through acidic regions, this suggested that co-operativity is a property intrinsic to such domains, and that the inability of E6−E2 to operate similarly reflects a potentially different effector mechanism.

The characteristic feature of all E6 proteins is their composition—rich in cysteine, arginine and lysine. Most of the cysteine residues are present in the motif Cys-x-x-Cys, where 'x-x' are two variable amino acids. The two pairs of these motifs present in E6 proteins have the potential to form two 'zinc fingers', and both BPV and HPV 18 E6 proteins have been reported to bind metal using an *in vitro* filter assay (Barbosa *et al.*, 1989; Grossman and Laimins, 1989). Levels of β-gal production did not increase when E6−E2 synthesizing yeast were supplemented with zinc (data not shown).

Three distinct biochemical functions have been attributed to cysteine-rich motifs. Present in two forms in several transcription factors, the fingers are believed to mediate sequence specific DNA binding (Mitchell and Tjian, 1989). One form, typified by TFIIIA and the transcription factor Sp1, has cysteine and histidine pairs that coordinate coupling of the metal ion to the peptide, while the other form, found in yeast and mammalian (e.g. steroid hormone) transcriptional activators, is composed of two pairs of cysteine residues at the base of the finger structure. In both the Cys/His and Cys/Cys models, the 'fingers' themselves usually include 12 amino acids, while E6 has ~30 residues. A second potential role for the finger structure is protein−protein interactions. In the HIV-1 *tat* protein, cysteine motifs are postulated to allow a metal based bridge between two monomers of *tat* (Frankel *et al.*, 1988). Thirdly, the cysteine-rich zinc finger region of protein kinase C has been implicated in the binding of phorbol ester (Ono *et al.*, 1989). The inability of E6−E2 cysteine mutations (Table III) at the stem of the putative E6 zinc fingers to induce β-gal production provided further evidence that *trans*-activation was specifically dependent on an E6 domain and inferred that the folding of E6 into a zinc finger conformation was a prerequisite for its transcriptional function. Therefore these findings suggest that the zinc finger motif may also function as a transcriptional activator. We are currently examining the requirement for each of the E6 fingers in control of gene expression and transformation, and whether these properties are provided by identical or distinct domains in E6.

Our data demonstrate that E6 can activate transcription when in proximity to promoter elements. This could be modulated by E6 binding to a specific DNA sequence, perhaps with a zinc finger. The transcriptional activity we describe will allow isolation of an E6 responsive element and the determination of its recognition by E6. Alternatively, it is possible that E6 could activate a specific DNA element without binding to DNA. For example, since E6 proteins are predicted to have two such 'fingers', one could interact with a sequence specific DNA binding protein and the other finger constitute a transcriptional activation domain. This resembles one hypothesis for adenovirus E1a function as a transcriptional factor, since it does not bind DNA directly, but when targeted to a promoter strongly induces mRNA synthesis (Lillie and Green, 1989). It is also possible that E6 modifies another transcription factor that in turn activates a specific DNA element, without requiring E6 at the promoter. While this model cannot be excluded, our data support a role for E6 at the transcriptional start site.

BPV E6 and E6−E2-R were compared for their ability to transform mouse cells using a focus formation assay. Similar efficiency was observed with the hybrid as with E6, but both failed to transform NIH 3T3 cells (data not shown). Since E6−E2 acted as a transcription factor in *S.cerevisiae*, this implied that it can conduct the events necessary for RNA production through an evolutionarily conserved pathway. Therefore it was unlikely that the transcription function we now assign to a domain in E6 would, in the context of the E6−E2 chimera, discriminate the critical differences between C127 and NIH 3T3 cells in susceptibility to transformation.

The HPV and animal papillomaviruses have been implicated in the etiology of benign and malignant epithelial tumors in their respective hosts. In particular, HPV types 16 and 18 are associated with malignant carcinoma of cervix and penis, while HPV types 6 and 11 are limited to benign disease. We are therefore examining the ability of HPV 16 E6 to *trans*-activate a promoter by cloning this reading frame onto the carboxy-terminus of BPV E2 in an analogous manner to BPV E6. Preliminary data indicate that HPV 16 E6 increases β-gal expression 5- to 8-times over basal levels in *S.cerevisiae*. E1a-like transcriptional activity has been reported for another HPV 16 gene, E7 (Phelps *et al.*, 1988). It is also a transforming gene, and is thought to mediate this through its binding to the retinoblastoma gene product (Dyson *et al.*, 1989). Several groups have reported that both HPV E6 and E7 are required for full transformation of primary keratinocytes *in vitro* (Hawley-Nelson *et al.*, 1989;

Munger *et al.*, 1989; Hudson *et al.*, 1990), and this suggests that E6 and E7 could also exhibit complementarity at the transcriptional level.

In summary, we have demonstrated that the zinc 'finger' BPV E6 protein encodes a domain that stimulates transcription when localized to a promoter, thus resembling several other nuclear oncogenes (Varmus, 1987). Further studies are required to determine the relevance of this transcriptional activity to its transforming function. We hypothesize that the zinc finger itself may form a novel activation domain that may be operationally distinct from the acidic amphipathic helix characteristic of other transcription factors, including BPV E2. We suggest that the E6 transcription function is common to BPV and HPV 16 and together with E7 may contribute to malignant transformation by specifically altering the transcriptional program of the cell.

Materials and methods

Constructions

BPV E6 was converted to a *Bam*HI cassette by cloning the *Hpa*II fragment [nucleotides (nt) 80–669] into the *Bam*HI site of pUC12 after both had been made flush ended with Klenow and dNTPs. This restored *Bam*HI ends and retained the first ATG codon in the reading frame. E6 and the E6–E2-R fragments from the pUC vector described below were subsequently cloned into the *Bam*HI site of the pZipNeoSV(X)-1 vector (kindly provided by R.Cone) or the URA based yeast expression vector pKP15 which included the GAL UAS and CYC-1 promoter, a high copy number 2μ replication origin and URA gene (Morrissey *et al.*, 1989).

The BPV E6–E2 fusion was constructed in the *Bam*HI site of pUC12 (pUCE6–E2) by linking the BPV E6 fragment (nt 80–480, *Bam*HI–*Pst*I) in frame to the BPV1 E2 fragment (nt 3089–4450, *Nco*I–*Bam*HI). This portion of E2 (called pYE2-R, Morrissey *et al.*, 1989) excluded the initial 160 amino acids of the E2 transcriptional activation region. E6 was joined to E2-R through double stranded oligonucleotides

GA CAT GGT TCA AGG TCC **AGG** TAC CC
ACGT CT GTA CCA AGT TCC AGG **TCC** ATG GG GTAC

that form *Pst*I and *Nco*I restriction overhangs at their ends. The oligonucleotides were designed to create a *Kpn*I site by replacing the wild-type codon AAA with AGG (bold in above), substituting lysine with arginine. This *Kpn*I site is out-of-frame with a unique *Kpn*I site in the 'hinge' region of E2 (amino acid 285, nt 3455), and thus digestion of E6–E2 with *Kpn*I and re-ligation of the ends created E6–E2-K. The terminal BPV E6 proline is lost in E6–E2-K and 22 amino acids of unrelated peptide are added to the carboxy-terminus of E6. After cleaving the *Kpn*I site in E6–E2-K with its isoschizomer *Asp*718, treatment with Klenow and dNTPs, and subsequent re-ligation, the E2 DNA binding domain was restored to the E6 reading frame (Figure 1). E6–E2-Kif therefore excluded amino acids 161–285 which included the residual E2 activation region present in E2-R and E6–E2-R.

An in-frame linker insertion mutation in the E2 DNA binding domain at nt 3812 (Haugen *et al.*, 1988) was transposed into the E6–E2 chimeric vectors by substituting the *Nco*I (nt 3089)–*Bst*XI (nt 3888) fragment from E2-3812 (provided by J.Schiller) into pUC E6–E2 (pUC E6–E2-3812). VP16-E2 was constructed by amplification of the carboxy-terminal 70 amino acids of HSV-1 VP16 (Sadowski *et al.*, 1988) with PCR primers and was subsequently cloned in-frame onto the carboxy-terminal 100 amino acid DNA binding domain of E2. AH-E2 was constructed by synthesis of complementary 50 bp oligonucleotides coding for a 15 amino acid amphipathic helix (Giniger and Ptashne, 1987) that allowed in-frame fusion at its 3′ end to this E2 DNA binding region. The integrity of each hybrid was confirmed by Western blot of protein produced in bacteria (VP16-E2 = 30 kd, AH-E2 = 16 kd; data not shown).

E6 mutants (Vousden *et al.*, 1989) were transferred from the LTR vectors after amplification with Taq polymerase and synthetic oligonucleotides that provided a 5′ *Bam*HI and a 3′ *Nco*I cloning site and flanked the E6 gene. Each was confirmed by restriction endonuclease mapping. These E6–E2-R chimeras retain the wild-type lysine in their carboxy-termini.

Trans-activation in mouse cells and in S.cerevisiae

The mammalian cell reporter plasmids contained one, two, three or two mutant E2 binding sites (Hawley-Nelson *et al.*, 1988), an enhancerless SV40 promoter and the CAT gene. Transfections were carried out by calcium phosphate co-precipitation using 2–5 μg of each plasmid and 5 μg of salmon sperm DNA. CAT activity was measured by thin layer chromatography after 48 h as previously described (Prakash *et al.*, 1988).

The LEU based yeast target plasmids pBY-1, -2 and -4 that contain one, two or four E2 binding sites upstream from the CYC-1 TATA elements and the *lacZ* gene were transformed into *S.cerevisiae* strain BGW1-7a (*leu*$^-$ *ura*$^-$) (Morrissey *et al.*, 1989). LEU$^+$ URA$^+$ colonies were initially amplified in glucose, and switched to galactose containing media 12 h prior to assay. The levels of *lacZ* expression were quantified by measuring the β-gal activity. Cells were cultured on minimal media plates with 2% galactose as carbon source and X-gal.

Acknowledgements

We appreciate the comments of V.Dias-Ferrao in the initial design of the E6–E2 plasmid, S.S.Prakash for helpful discussions, J.Schiller and R.Cone for plasmids, and L.Laimins for review of the manuscript. This work was supported by grants from the National Cancer Institute.

References

Androphy,E.J., Schiller,J.T. and Lowy,D.R. (1985) *Science*, **230**, 442–445.
Androphy,E.J., Hubbert,N.L., Schiller,J.T. and Lowy,D.R. (1987a) *EMBO J.*, **6**, 989–992.
Androphy,E.J., Lowy,D.R. and Schiller,J.T. (1987b) *Nature*, **324**, 70–73.
Barbosa,M.S., Lowy,D.R. and Schiller,J.T. (1989) *J. Virol.*, **63**, 1404–1407.
Bedell,M.A., Jones,K.H. and Laimins,L.A. (1987) *J. Virol.*, **61**, 3635–3640.
Cepko,C.L., Roberts,B.E. and Mulligan,R.C. (1984) *Cell*, **37**, 1055–1062.
Cerni,C., Binetruy,B., Schiller,J.T., Lowy,D.R., Meneguzzi,G. and Cuzin,F. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3266–3270.
Cole,S.T. and Danos,O. (1987) *J. Mol. Biol.*, **193**, 599–608.
Courey,A.J. and Tjian,R. (1988) *Cell*, **55**, 887–898.
Curran,T. and Franza,B.R.,Jr (1989) *Cell*, **55**, 395–397.
Dynan,W.S. (1988) *Cell*, **58**, 1–4.
Dyson,N., Howley,P.M., Munger,K. and Harlow,E. (1989) *Nature*, **243**, 934–937.
Frankel,A.D, Bredt,D. and Pabo,C.O. (1988) *Science*, **240**, 70–73.
Giniger,G.M. and Ptashne,M. (1987) *Nature*, **330**, 670–672.
Giri,I. and Yaniv,M. (1988) *EMBO J.*, **7**, 2823–2829.
Gius,D., Grossman,S., Bedell,M.A. and Laimins,L.A. (1988) *J. Virol.*, **62**, 665–672.
Grossman,S. and Laimins,L. (1989) *Oncogene*, **4**, 1089–1093.
Grossman,S., Mora,E. and Laimins,L. (1988) *J. Virol.*, **63**, 366–374.
Haugen,T.H., Cripe,T.P., Ginder,G.D., Karin,M. and Turek,L.P. (1987) *EMBO J.*, **6**, 145–152.
Haugen,T.H., Turek,L.P., Mercurio,F.M., Cripe,T.P., Olson,B.J., Anderson,D.S., Karin,M. and Schiller,J. (1988) *EMBO J.*, **7**, 4245–4253.
Hawley-Nelson,P., Androphy,E.J., Lowy,D.R. and Schiller,J.T. (1988) *EMBO J.*, **7**, 525–531.
Hawley-Nelson,P., Vousden,K.H., Hubbert,N.L., Lowy,D.R. and Schiller,J.T. (1989) *EMBO J.*, **8**, 3905–3910.
Hudson,J.B., Bedell,M.A., McCance,D.J. and Laimins,L.A. (1990) *J. Virol.*, **64**, 519–526.
Iftner,T., Bierfelder,S., Csapo,Z. and Pfister,H. (1988) *J. Virol.*, **62**, 3655–3661.
Lambert,P.F., Spalholz,A. and Howley,P.M. (1987) *Cell*, **50**, 68–78.
Lambert,P.F., Dostatni,N., McBride,A.A., Yaniv,M., Howley,P.M. and Arcangioli,B. (1989) *Genes Dev.*, **3**, 38–48.
Lech,K., Anderson,K. and Brent,R. (1988) *Cell*, **52**, 179–184.
Lillie,J.W. and Green,M.R. (1989) *Nature*, **338**, 39–44.
Lillie,J.W., Loewenstein,P.M., Green,M.R. and Green,M. (1987) *Cell*, **50**, 1091–1100.
Ma,J. and Ptashne,M. (1987) *Cell*, **51**, 113–119.
Mallon,R.G., Wojciechowicz,D. and Defendi,V. (1987) *J. Virol.*, **61**, 1655–1660.

McBride,A.A., Schlegel,R. and Howley,P.M. (1988) *EMBO J.*, **7**, 533–539.
McBride,A.A., Byrne,J.C. and Howley,P.M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 510–514.
Mitchell,P.J. and Tjian,R. (1989) *Science*, **245**, 371–378.
Mermod,N., O'Neill,E.A., Kelly,T.J. and Tjian,R. (1989) *Cell*, **58**, 741–753.
Morrissey,L., Barsoum,J. and Androphy,E.J. (1989) *J. Virol.*, **63**, 4422–4425.
Munger,K., Phelps,W.C., Bubb,V., Howley,P.M. and Schlegel,R. (19897 *J. Virol.*, **63**, 4417–4421.
Ono,Y., Fujii,T., Igarashi,K., Kuno,T., Tanaka,C., Kikkawa,U. and Nishizuka,Y. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4846–4871.
Phelps,W.C., Yee,C.L., Munger,K. and Howley,P.M. (1988) *Cell*, **53**, 539–547.
Prakash,S.S., Horowitz,B.H., Zibello,T., Settleman,J. and DiMaio,D. (1988) *J. Virol.*, **62**, 3608–3613.
Ptashne,M. (1988) *Nature*, **335**, 683–689.
Sadowski,I., Ma,J., Triezenberg,S. and Ptashne,M. (1988) *Nature*, **335**, 563–564.
Schiller,J.T., Vass,W.C. and Lowy,D.R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7880–7884.
Spalholz,B.A., Yang,Y. and Howley,P.M. (1985) *Cell*, **42**, 183–191.
Varmus,H.E. (1987) *Science*, **238**, 1337–1339.
Vousden,K.H., Androphy,E.J., Schiller,J.T. and Lowy,D.R. (1989) *J. Virol.*, **63**, 2340–2342.
Watanabe,S., Kanda,T. and Yoshiike,K. (19897 *J. Virol.*, **63**, 965–969.

Received on February 19, 1990