Virology **270,** 430–443 (2000)
doi:10.1006/viro.2000.0257, available online at http://www.idealibrary.com on IDEAL®

The Bovine Papillomavirus E2 Transactivator Is Stimulated by the E1 Initiator through the E2 Activation Domain

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Received November 10, 1999; returned to author for revision December 20, 1999; accepted February 15, 2000

Bovine papillomavirus type 1 (BPV-1) encodes two regulatory proteins, E1 and E2, that are essential for viral replication and transcription. E1, an ATP-dependent helicase, binds to the viral *ori* and is essential for viral replication, while the viral transcriptional activator, E2, plays *cis*-dominant roles in both viral replication and transcription. At low reporter concentrations, E1 stimulates E2 enhancer function, while at high reporter concentrations, repression results. An analysis of *cis* requirements revealed that neither replication nor specific E1-binding sites are required for the initiators' effect on E2 transactivator function. Though no dependence on E1-binding sites was found, analysis of E1 DNA binding and ATPase mutants revealed that both domains are required for E1 modulation of E2. Through the use of E2 fusion-gene constructs we showed that a heterologous DNA-binding domain could be substituted for the E2 DNA-binding domain and this recombinant protein remained responsive to E1. Furthermore, E1 could rescue activation domain mutants of E2 defective for transactivation. These data suggest that E1 stimulation of E2 involves interactions between E1 and the E2 activation domain on DNA. We speculate that E1 may allosterically interact with the E2 activation domain, perhaps stabilizing a particular structure, which increases the enhancer function of E2.

INTRODUCTION

The bovine papillomavirus (BPV) transformation process has been an interesting model for understanding how transcription and replication occur in higher eukaryotes and how these aspects of DNA function may be coordinately regulated. Latently, the viral genome replicates as a stable nuclear plasmid in synchrony with the host cell cycle, requiring host cell machinery for both its replication and gene expression. The key events of the BPV plasmid state are regulated by two virally encoded proteins, the E1 initiator protein and the transcriptional activator, E2. Replication of BPV, *in vivo* and *in vitro,* requires the E1 protein. This protein recognizes the viral origin as a monomer but in an ATP-dependent step E1 oligomerizes to form a functional helicase that binds the origin of replication (*ori*) as a hexamer and interacts with cellular DNA polymerase α-primase and the cyclin E–cdk2 complex (Cueille *et al.,* 1998; Fouts *et al.,* 1999; Sedman and Stenlund, 1998; Stenlund, 1996). Replication of BPV *in vivo* also requires the E2 protein (Ustav and Stenlund, 1991), while *in vitro,* E2 functions as a replication enhancer stimulating DNA replication and is only absolutely required for DNA replication at limiting concentrations of the E1 protein (Yang *et al.,* 1991a).

The E2 transactivator has a modular structure containing an amino-terminal activation domain and a carboxy-terminal DNA-binding and dimerization domain separated by an unstructured "hinge" region (McBride *et al.,* 1989). The E2 ORF encodes the full-length E2 transactivator, as well as two amino-terminal truncations, E2C and E8E2, that lack the activation domain and act as transcriptional and replication repressors (Choe *et al.,* 1989; Hubbert *et al.,* 1988; Lambert and Howley, 1988; Lim *et al.,* 1998). E2 interacts directly with E1, and the two proteins bind cooperatively to DNA (Mohr *et al.,* 1990). It is this cooperative binding that mainly explains E2's replication enhancer activity, as the cooperativity is required for targeting the E1 monomers to the origin site under physiological conditions. Relative to the full-length forms, the two repressor forms of E2 do not cooperatively interact with the E1 replication factor (Lim *et al.,* 1998; Mohr *et al.,* 1990). Mutational analysis of E2 also indicates that the major surface of E2 that interacts with E1 is the amino-terminal activation domain (Benson and Howley, 1995; Ferguson and Botchan, 1996; Winokur and McBride, 1996). There is now compelling evidence that the E2 DNA-binding domain also interacts with E1, albeit at the DNA site weakly, and that this interaction is important for development of a preinitiation complex (Berg and Stenlund, 1997; Chen and Stenlund, 1998).

The role of the E2 transactivator as a specificity factor for targeting E1 to the origin site has been firmly estab-

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0042-6822/00 $35.00

lished (Mohr *et al.,* 1990; Sedman and Stenlund, 1995, 1998; Sedman *et al.,* 1997; Ustav and Stenlund, 1991; Yang *et al.,* 1991a). In the presence of both E1 and E2 proteins, strong E2-binding sites can compensate for mutations in the E1 origin-binding site as judged by DNA-binding assays *in vitro* and in replication assays both *in vivo* and *in vitro* (Sedman and Stenlund, 1996; Spalholz *et al.,* 1993). Reciprocally, it was demonstrated that E1 mutants crippled for DNA binding can be rescued for binding *in vitro* by addition of E2 and, in some cases, can be rescued for their ability to promote replication *in vivo* (Thorner *et al.,* 1993). The central role of E2 in regulating viral DNA functions is further illustrated by the fact that the transactivator serves to tether the BPV genome to cellular chromosomes, providing a mechanism for maintenance of BPV as a nuclear plasmid (Ilves *et al.,* 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). Second-site suppressor mutations of the E2 phosphorylation mutant defective in plasmid maintenance were found in both E2 and E1, suggesting a potential role for E1 in plasmid maintenance (Lehman and Botchan, 1998).

Several lines of evidence suggest a role for E1 in modulating E2-dependent transactivation, including the organization of the BPV regulatory region, commonly known as the upstream regulatory region (URR). BPV E2 and its two repressor forms, E2C and E8E2, bind to 17 sites within the viral genome, 12 of which are located in the URR of the virus (Li *et al.,* 1989). Two weak E2-binding sites flank the viral *ori,* while 4 strong E2-binding sites that make up the E2 responsive element, E2RE1, also flank an E1-binding site (Mendoza *et al.,* 1995; Yang *et al.,* 1991b). The BPV *ori* is located 89 bp 5′ to the P_{89} viral mRNA start site and is also coincident with the P_{7940} promoter (Fig. 1A). Transcription from one or both of these promoters may be affected by E1 binding to the *ori,* by replication, or by binding of a multinucleoprotein complex to the site. This possibility is intriguing, since E1 itself may be expressed from one of these promoters. Although this concept has not been established, it is known that this cluster of start sites is the most active promoter region upstream of the E1 ORF in transformed cells (Baker and Howley, 1987; Choe *et al.,* 1989; Stenlund and Botchan, 1990; Stenlund *et al.,* 1985).

Genetic evidence also suggests a role for E1 in modulating E2 transactivator function. Experiments in which either the E1 or the E2 gene in the viral genome of HPV-16 is incapacitated show increased transformation efficiencies, consistent with the notion that both genes can negatively regulate, either directly or indirectly, expression levels of the viral oncogenes E6 and E7 (Romanczuk and Howley, 1992). In HPV-derived cervical carcinoma cell lines, HPV is usually integrated into the host chromosome, in a manner that usually disrupts either the E1 or the E2 gene products, thus leading to increased levels of viral oncogene expression (Durst *et al.,* 1985; Matsukura *et al.,* 1989; Schneider-Maunoury *et al.,* 1987; Shirasawa *et al.,* 1988). It is clear, however, that the transcriptional programs of the genital HPVs and BPV-1 are not completely analogous. For the major viral promoters in the BPV-1 case, E2 serves as an activator, whereas for those human papillomaviruses analyzed, E2 negatively modulates the major promoters (Howley, 1996). It is important to point out that some of the differences may be more quantitative than qualitative in nature as a report shows that the HPV11 E2 can activate a major viral promoter at low concentrations of E2 and switches to a repressor at high levels of protein as more E2 *cis*-acting elements are occupied (Chin *et al.,* 1989).

A survey of the BPV-1 literature on the effects of E1 upon E2 activation finds the issue rather unresolved. Several laboratories have reported genomic mutants in BPV E1 and E2 that have effects on transformation, suggesting that replication or transcription, or a combination thereof, could be involved in regulating the expression of viral oncogenes. In some reports, E1 genomic mutants show increased transformation efficiency, and significantly higher expression from viral promoters, when adjusted for template concentration (Lambert and Howley, 1988; Schiller *et al.,* 1989; Vande Pol and Howley, 1995). These reports postulate that E1 down-regulates the E2 transcriptional regulatory circuit, and therefore, its loss of function leads to enhanced transcription from viral promoters. Other groups have reported E1 genomic mutants with reduced transformation capabilities (Chiang *et al.,* 1992a; Lusky and Botchan, 1985; Sun *et al.,* 1990). In agreement with an interpretation placing the primary effect of these mutations on viral replication, RNA analysis of mutant viral genomes transiently expressed in C127 showed no effect of the E1 mutations on any viral promoters (Szymanski and Stenlund, 1991).

Several reports have also directly addressed the role of E1 in modulating E2 enhancer function in transient cell-based assays, although differences in the effect of E1 on E2 function were again obtained (Ferran and McBride, 1998; Le Moal *et al.,* 1994; Sandler *et al.,* 1993; Szymanski and Stenlund, 1991). One group (Szymanski and Stenlund, 1991) observed no effect of E1 on viral promoter activity, while another report showed E1 capable of stimulating E2 enhancer function (Le Moal *et al.,* 1994), while yet others reported E1 repression of E2 transactivation (Ferran and McBride, 1998; Sandler *et al.,* 1993). Differences in the cell type and experimental design have probably contributed to these disparate results. In addition, a variety of viral nuclear protein complexes can form over the *ori* region, some perhaps with different (or opposite) effects on transcription, and the types of such complexes are determined by the levels of the E1 and E2 proteins that may vary in different experimental designs.

Here we address the role of E1 in E2 transactivation in

an attempt to clarify some of the discrepancies in the field, to extend our understanding of the role of E1 in E2 enhancer function. As one approach to this problem, a variety of *cis* and *trans* mutants, whose phenotypes in certain assays have been previously characterized, were systematically used to elucidate important features involved in E1 modulation of E2 enhancer function.

Employing reporters, some of which were defective for DNA replication, in a cell-based transient transfection assay, we determined that E1 does modulate E2 transactivator function. Under conditions where E1 stimulates E2 transactivation, we determined that the E1 DNA-binding domain was important for stimulation of E2 enhancer function, although a specific E1 DNA-binding site was not important for this effect. Through the use of E1 mutants, we found that multiple domains of E1 are required to stimulate E2 enhancer function. We also provide evidence consistent with the notion that interactions between E1 and the E2 activation domain are important for such synergy between the two viral proteins for transcription function. E1 stimulated E2 enhancer function when E2 was fused to a heterologous DNA-binding domain, although it would not stimulate a heterologous activation domain fused to the E2 DNA-binding domain. Furthermore, we show here that E1 rescues E2 activation domain mutants that are defective for transactivation. Our data are also consistent with a negative regulatory role for E1 under certain proscribed conditions.

RESULTS

E1 stimulates E2 enhancer function in bovine embryo fibroblasts

The role of the BPV replication protein E1 in E2-dependent transcription was addressed using *in vivo* luciferase assays performed in a bovine embryo fibroblast (BEF) primary cell line. Cells were transfected with a mixture of DNAs containing the reporter construct URR–Luc, as well as vectors that expressed E2 and E1, from the heterologous cytomegalovirus (CMV) promoter (Ustav and Stenlund, 1991). An E1 translation construct with a termination linker (E1TTL) inserted in the 5′ end of the E1 ORF (Fig. 1A) was used as a negative control for E1. The reporter, URR–Luc, contains several viral promoters, P_{7185}, P_{7940}, and P_{89}, the BPV *ori,* the major enhancer element E2RE1, and many other previously characterized E2-binding sites that make up the URR (Haugen *et al.,* 1987; Li *et al.,* 1989; Spalholz *et al.,* 1985, 1987) fused to the firefly luciferase gene. Transfection of the reporter and E2 alone demonstrated that E2 could stimulate transcription from this reporter construct. Light activity of URR–Luc transfected with E2 was approximately 4.8 arbitrary light units compared to 0.01 light units obtained with URR–Luc alone. As shown in Fig. 1B, increasing the amounts of pCG-E1 transfected into cells containing 250 ng of the URR–Luc and 50 ng of pCG-E2 increased the luciferase activity as measured in arbitrary light units, showing up to eightfold stimulation of light activity over that obtained with E2 alone. A similar titration of the control expression vector, pCG-E1TTL, resulted in no stimulation of light activity over E2 stimulation alone. Only 0.02 arbitrary light units of luciferase activity were obtained from cell lysates when either pCG-E1 or pCG-E1TTL was transfected into cells with the reporter plasmid alone (data not shown). These data indicate that E1 specifically stimulates E2-dependent transcription from the URR–Luc reporter, but is not, by itself, acting as a transcription factor. These gene expression results were further substantiated by extraction of total RNA from transfected cells and direct primer extension analysis (Fig. 1C). The P2CAT reporter construct was used (Stenlund and Botchan, 1990) instead of URR–Luc, due to increased stability of the message. We found that the P_{89} promoter was indeed activated by E1, being readily detected only when E1 and E2 were coexpressed.

URR–Luc, which contains the BPV *ori,* replicates in the presence of both E1 and E2 in BEF cells (data not shown). Thus, the increase in luciferase activity could have been due to amplification of the reporter template, rather than to a direct effect of E1 on E2 enhancer function. Therefore, we asked whether the stimulation of transcription could be seen in the absence of replication by using a reporter construct, E2RE1tk-Luc, incapable of replication. Although E2RE1tk-Luc contains both E1- and E2-binding sites, as determined by DNA protein coimmunoprecipitation experiments and DNase 1 protection experiments (Mendoza *et al.,* 1995; Mohr *et al.,* 1990), it does not replicate *in vivo* as it contains no bona fide *ori* (data not shown).

E2 stimulated transcription from this reporter, giving a light activity of 30 arbitrary light units in this experiment, while in the absence of E2, light activity from reporter alone was about 0.3 light units. The presence of pCG-E1 or pCG-E1TTL did not significantly affect this basal reporter activity in the absence of E2 (data not shown). A titration of increasing amounts of pCG-E1 transfected with 50 ng of pCG-E2 and 250 ng of E2RE1tk-Luc resulted in an increase of light activity, about sevenfold, over that seen with E2 alone (Fig. 1D). As a comparison, no detectable increase in light activity was seen when pCG-E1TTL was titrated in the presence of E2 expression vector and reporter. Although the overall activity of the E2RE1tk-Luc reporter was higher than that seen with URR–Luc, both in the presence and in the absence of E2, qualitatively, the results are thus similar. The stimulation by E1 of E2-dependent gene expression from these two reporter constructs indicates that replication of the URR–Luc reporter does not significantly contribute to the stimulatory effect of E1 on E2 enhancer function. Other experiments, described later, further substantiate the point that replication is not a significant factor in E1 stimulation of E2 enhancer function.

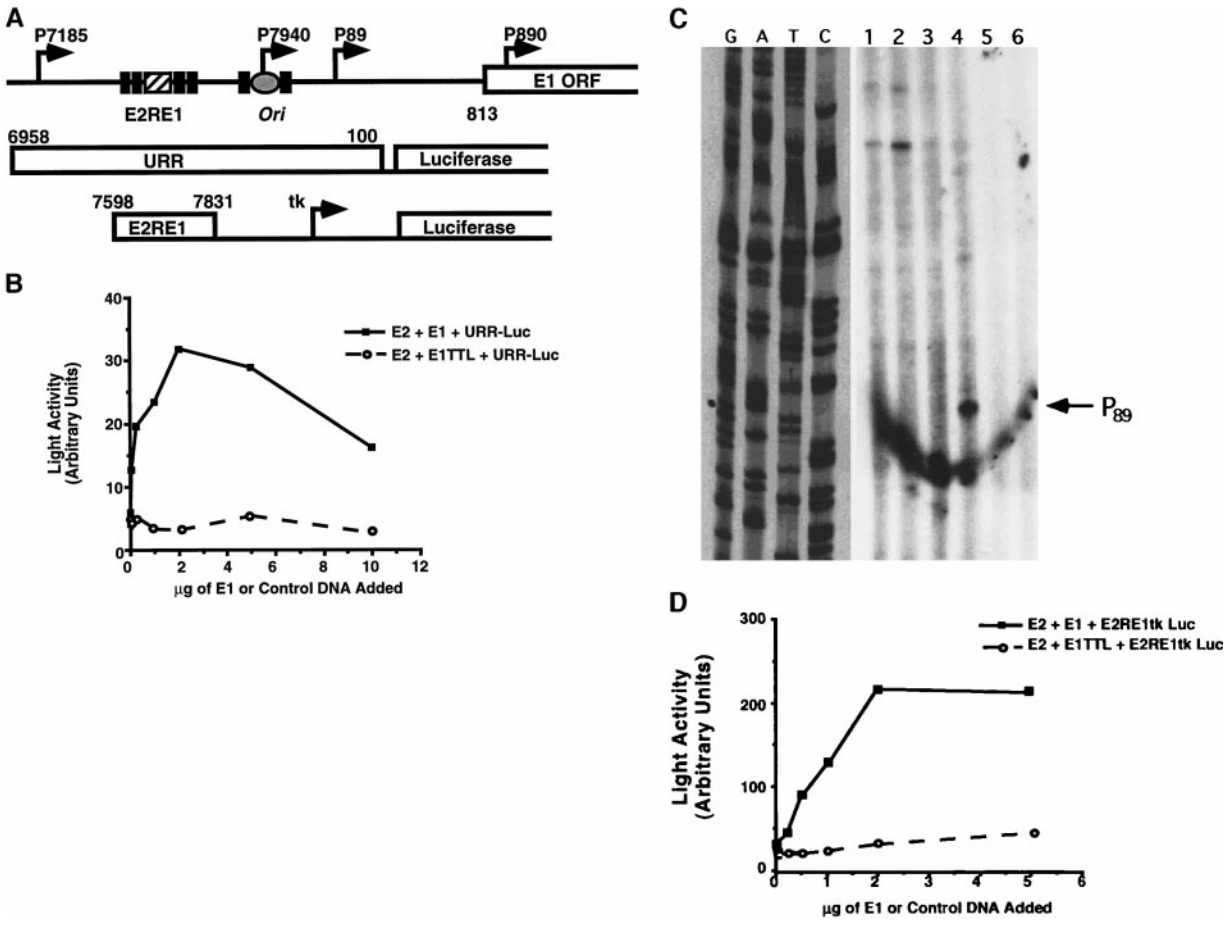

FIG. 1. (A) Schematic of the upstream regulatory region. URR of BPV contains E2 binding sites (black boxes) flanking the E1-binding sites (striped box and oval) at the enhancer element E2RE1 and the BPV *ori* (oval). Viral promoters P_{7185}, P_{7940}, P_{89}, and P_{890} are depicted with arrows. P_{7940} and P_{89} are located proximal to the binding sites in the BPV *ori* and upstream of the E1 ORF, which begins at nucleotide 813. P_{890} is within the E1 coding region. URR luciferase contains BPV sequences from 6958 to 100 nt upstream of the luciferase gene, while E2RE1tk-luciferase contains BPV nucleotides 7598–7831 upstream of the minimal thymidine kinase gene promoter upstream of luciferase. (B) Depicted is a representative experiment of BEF cells transfected with 50 ng of pCG-E2 and increasing amounts of pCG-E1 (solid line) or E1TTL (broken line) in the presence of 250 ng of the replicating URR–Luc reporter or the nonreplicating E2RE1tk-Luc reporter (D). Cells were electroporated and harvested after 48 h and assayed for luciferase activity as described elsewhere. Experiments were done in duplicate a minimum of three times. (C) Primer extension analysis of P2CAT transcripts was performed with total RNA isolated from BEF cells 2 days after electroporation. The P_{89} extension product is denoted. Lane 1 shows mock-transfected cells. Lanes 2 through 6 were transfected with 250 ng P2CAT. Lane 3 was additionally transfected with 50 ng pCG-E2, lane 4, with 50 ng pCG-E2 as well as 5 μg pCG-E1, lane 5, with 50 ng pCG-E2 and 5 μg pCG-E1TTL, and lane 6 with pCG-E1. A primer extension product corresponding to the predicted size of P_{89} can be seen in the presence of both E2 and E1. A sequencing ladder of P2CAT using the same primer is shown on the right, to identify the 5′ extension product of the P_{89} transcript.

Stimulation of E2 transactivation by E1 is dependent on reporter concentration

Variables were sought that might explain some of the discrepancies between the different published reports and the data presented here. One difference between the different reports was the concentration and type of reporter construct used. Because both E1 and E2 are DNA-binding proteins that interact with each other and DNA as multimers, reporter concentration and thus the effective protein-binding site ratio could play a critical role in the effect of E1 on E2 enhancer function. This is in part because the E2 transactivator function is synergistic and E2 dimers bound to multiple E2-binding sites upstream of a promoter have significantly greater ability to stimulate transcription than does a single E2 dimer bound to a site upstream of a promoter (Li *et al.,* 1991).

To address the potential role of reporter concentration on E1 stimulation of E2 enhancer function, transfections in BEFs were performed in which the levels of E1 and E2 expression vector DNAs were held constant, while the amount of the reporter varied. Experiments were performed with 50 ng of pCG-E2 and 1 or 5 μg of pCG-E1 (5 μg shown) or pCG-E1TTL as a negative control. These expression vectors were transfected into cells with increasing amounts of the URR–Luc reporter construct, and reporter gene activity was measured. The level of E1 stimulation of E2 enhancer function was typically high at low reporter concentrations (see inset, Fig. 2A). As re-

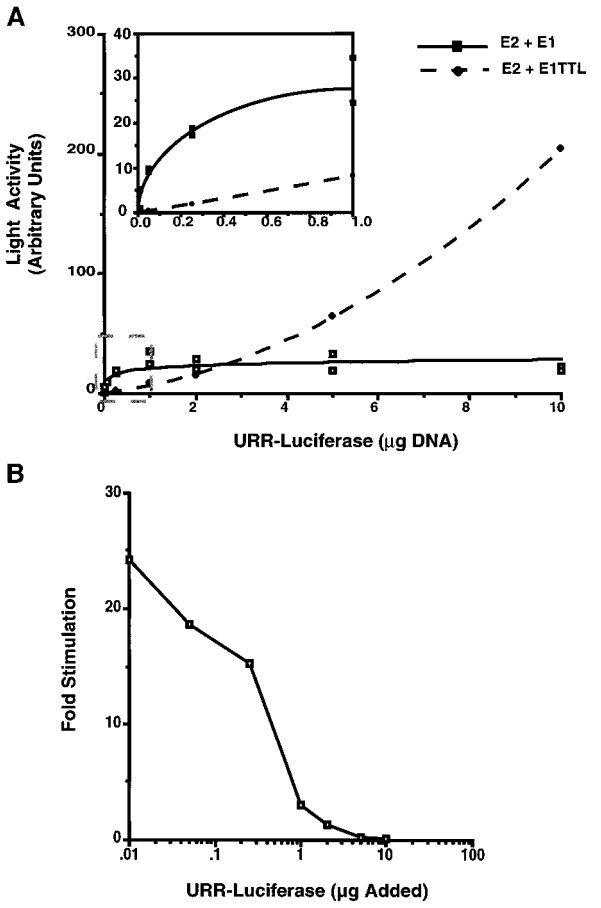

FIG. 2. E1 modulates E2 enhancer function in a reporter concentration-dependent manner. In a representative experiment, 50 ng of pCG-E2 and 5 μg of pCG-E1 (solid lines) or E1TTL (broken lines) was transfected into cells with increasing amounts of the URR–Luc reporter (A and B). Duplicate points are depicted. Reporter gene expression is measured in arbitrary light units. The inset in A is an expansion of the data at low reporter concentrations. Graph of the fold effect of E1 on E2 transactivation is depicted in (B). Fold effect was determined by graphing the ratio of average light activity obtained from the duplicate samples at each concentration of reporter of E2 + E1/E2 + E1TTL.

porter concentration is increased, stimulation of transcriptional activation is reduced in the presence of E1 expression, with repression of E2 transactivation being detected at very high reporter concentrations (Fig. 2A). A graph of the E1 fold stimulation clearly depicts this trend (see Fig. 2B). E1 fold stimulation is maximal at the lowest reporter concentrations used, and the fold stimulation decreases as the reporter concentration is increased. Thus at high levels of reporter, E1 expression does indeed repress E2 activation. Similar results were obtained with the nonreplicating E2RE1tk-Luc reporter (data not shown). The repression we see at high reporter concentrations is consistent with published reports documenting E1 repression; however, the magnitude of the effect is not as dramatic (2- to 5-fold compared to 10- to 20-fold) (Sandler *et al.,* 1993).

This variation of E1's effect on E2 enhancer function in response to reporter concentration perhaps explains some of the discrepancies in the literature. Why does such reporter concentration dependence exist? It is known that E1 and E2 bind DNA cooperatively and that E2 stimulation of transcription is optimal when E2 acts as a multimer. Cooperative interactions between E1 and E2 may enhance E2 activity at low reporter concentrations, as the relative affinity of E2 for DNA is increased due to the presence of E1. In the presence of high reporter concentrations, E1 may have a distributive effect, bringing E2 to bind to too many different reporter molecules in the cell. This generalized distribution of E2 may effectively reduce the ability of E2 to multimerize, which may result in an apparent repression of transcription at high reporter concentrations.

E1 binding to a specific site is not required for stimulation of E2 enhancer function

We used previously characterized mutations in the BPV URR that disrupt E1 DNA binding to determine the role of specific E1-binding sites on E2 enhancer function. A series of E1-binding site mutations within the BPV *ori* and within E2RE1 were constructed in the URR–Luc reporter construct. For the 5C-20C series, linkers of increasing length in steps of 5 bp were inserted at the *ori* palindrome and the others are simple deletion mutations. Another reporter (d60dOpaI) was employed, containing a double mutant that eliminated E1-binding sites both in the *ori* and in E2RE1, which are absent in this construct. Previous studies demonstrated that mutations in these two regions severely alter, or eliminate, the E1 DNA footprints on these regions (Mendoza *et al.,* 1995). These studies also demonstrated that *in vivo,* the *ori* linker insertion mutants were severely impaired for replication, while the *ori* deletion mutant (dOpaI) completely abolished replication *in vivo*.

These mutant reporters were tested, under conditions in which E1 stimulates E2 enhancer function, to determine whether E1 would stimulate E2-dependent transcription in the absence of a known E1-binding site. Basal expression from these reporters was measured and was consistently low, with an average luciferase activity of 0.2 light units. Furthermore, pCG-E1 and pCG-E1TTL had no discernible effect on reporter light activity in the absence of E2 (data not shown). E2 activation of these reporters in the absence of E1 was slightly variable; notably, the *ori* linker insertion mutant 20C URR–Luc consistently exhibited lower activation by E2 than did the wild-type URR–luc reporter (by about half). The increased distance of the ori proximal E2-binding sites to one another may be responsible for this difference in transcriptional activity. E1 stimulated E2-dependent transactivation from each of these reporters. The data presented are averaged from 6–10 independent experi-

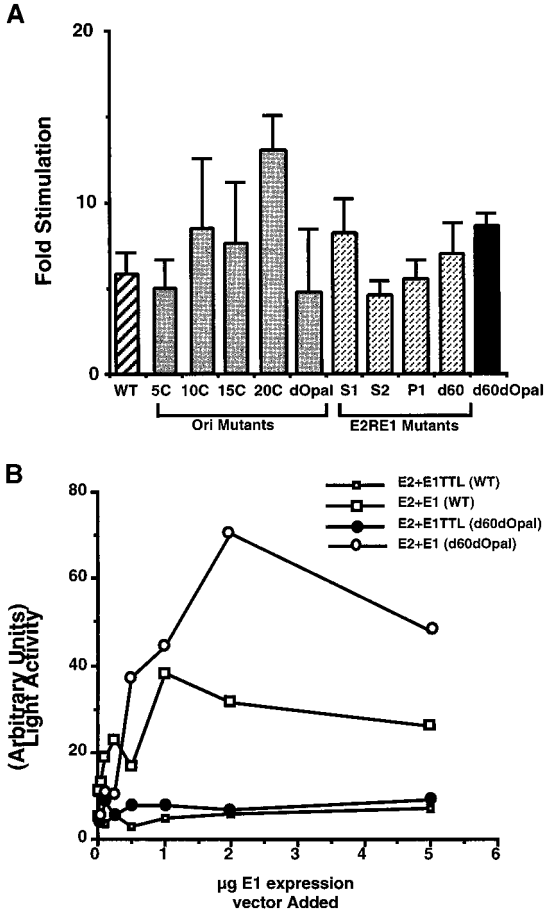

FIG. 3. An E1-binding site is not required for its stimulation of E2 enhancer function. (A) 50 ng of pCG-E2 was transfected into BEF cells in the presence of 5 μg of pCG-E1 or E1TTL with 250 ng of the wild-type or mutant URR-Luc reporter (as noted in graphs). 5C, 10C, 15C, and 20C are linker insertions at the BPV *ori* and dOpal is a deletion of the BPV *ori.* S1, P1, and S2 are substitution mutations of the E1-binding site in E2RE1, while d60 deletes the 60-bp element of E2RE1 where E1 footprints (Mendoza *et al.,* 1995). The stimulation of E1 on E2 enhancer function was determined by dividing E2 + E1/E2 + E1TTL for each mutant and is graphed here as fold stimulation. These data represent the average of 6–10 independent electroporations, each done in duplicate, and error bars represent 1 standard deviation. Depicted is the fold stimulation of E2 enhancer function by E1 of wild-type URR–Luc (striped column), origin mutant reporters (gray columns), E2RE1 mutant reporters (hatched columns), and a double deletion lacking E1-binding sites in both regions (black column). (B) Titrations with increasing amounts of pCG-E1 (open circles and squares) or pCG-E1TTL (filled circles and squares) performed in the presence of 50 ng of the E2 expression vector and 250 ng of the wild-type (solid lines and squares) or d60dOpal-mutant (broken lines and circles) URR–Luc reporter. Similar results were obtained from three additional independent experiments.

ments (Fig. 3A). Mutations in the E1-binding sites, including the double deletion mutant d60dOpal URR–Luc, had no effect on the ability of E1 to stimulate E2 enhancer function. The *ori* linker insertion mutants, as well as dOpal, the *ori* deletion, show significantly reduced levels of replication (Mendoza *et al.,* 1995), while they all behave equivalent to wild type in our assays here. Together, these data demonstrate that E1 stimulates E2 enhancer function in the absence of a specific E1-binding site.

In the analysis of E1 stimulation of E2 transactivation described above, the concentrations of the E1 expression vector used to compare the phenotypes of different *cis* mutants in the URR–Luc reporter were those that gave maximal effects, as determined by our initial titration experiments. It has been reported that the *in vitro* E1-binding affinity to nonspecific DNA is only 10- to 100-fold lower than its binding to specific DNA sequences (Sedman and Stenlund, 1996). It was thus possible that saturating amounts of E1 were able to obscure the potential role of a specific E1-binding site in regulating E2 enhancer function. To test this possibility, titrations were performed *in vivo* with increasing amounts of pCG-E1 and the total expression vector concentration was held constant, with E1TTL added to adjust for changes in the concentration of the E1 expression vector. The URR–Luc and the double deletion mutant (d60dOpal) were used as reporters in this analysis.

E1 stimulation of the wild-type reporter luciferase activity, URR–Luc, increased in a monotonic way with increasing E1 expression vector transfected into cells in the presence of E2. Similar increases were seen when E1 was titrated with the double E1-binding site deletion reporter, d60dOpalURR-Luc (Fig. 3B). Thus, the E1 site deletion mutant had no significant effect on E1 stimulation of E2 enhancer function at any concentration of E1 used relative to the wild-type reporter, and even at subsaturating levels of E1, specific E1-binding sites are not required to mediate E1 stimulation of E2 enhancer function.

Multiple domains of E1 are required for cooperative interactions with E2

We were interested in correlating any known functions of E1 with its ability to stimulate the E2 enhancer function using our transient transcription assay. To do this we took advantage of several well-characterized mutants of E1 that perturb the DNA-binding, cooperative DNA-binding with E2, ATP-binding, or ATP hydrolysis activities of E1.

Several of our previously characterized DNA-binding domain mutants, LPM3 (P266A, I268A, L275A), LPM4 (K241A, R243A), and LPM6 (R180A), are reduced substantially in their ability to bind an *ori*-containing fragment of DNA both in the presence and in the absence of E2. LPM1 (L275P) binds better than wild-type E1 *in vitro,* in the presence of E2, but its DNA binding is reduced in the absence of E2. LPM5 (R247A) binds DNA at significantly reduced levels from wild-type E1, both in the presence and in the absence of E2; however, its ability to bind DNA is stimulated by E2, albeit to a lesser extent than wild-type E1. A deletion mutant (d50) that deletes amino acids 250 to 300 of E1, part of the core region of the E1

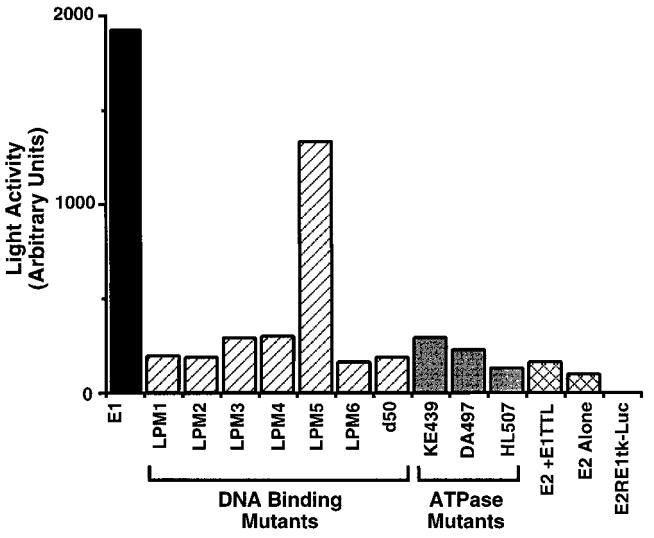

FIG. 4. Multiple domains of E1 are required to stimulate E2 enhancer function. 50 ng of pCG-E2 was transfected into BEF cells with 250 ng E2RE1tk-Luc reporter and 5 μg of wild-type or mutant E1 expression vectors as indicated, except for the far column, which contains reporter alone as a negative control. DNA-binding mutants (striped columns) and ATP mutants (gray columns) that fail to stimulate E2 are comparable to E2 + E1TTL or E2 alone (crossed columns) in their luciferase activity. LPM5, which has replication activity *in vivo,* is able to stimulate E2 in transactivation, although E2RE1tk-Luc does not replicate *in vivo.* Similar results were obtained from six additional independent electroporations, as well as six independent electroporations using 1 μg of the E1 expression vectors.

DNA-binding domain, has also been shown to be incompetent to bind DNA *in vitro.* Only one of these mutants, LPM5, is competent for replication (about 30% of wild-type levels), although all are expressed well *in vivo* (Thorner *et al.,* 1993).

Three E1 ATPase domain mutants, KE439, DA497, and HL507, were also assayed for their ability to stimulate E2 transcription. These mutants bind ATP at significantly reduced levels, are unable to hydrolyze ATP, and are unable to replicate a BPV *ori*-containing plasmid *in vivo* although they are, with the exception of KE439, similarly expressed (MacPherson *et al.,* 1994) and have been reported to bind DNA better than wild-type E1 (Thorner *et al.,* 1993).

These DNA-binding and ATPase mutants of E1 were tested for their ability to stimulate E2-dependent transcription *in vivo.* These E1 mutants had no effect on reporter activity in the absence of E2 (data not shown). We found that only one mutant, LPM5, is able to stimulate E2 enhancer function to nearly wild-type levels from both the replication-competent URR-Luc reporter (data not shown) and the non-replication-competent E2RE1tk-Luc reporter (see Fig. 4). These data are consistent with the notion that an intact DNA-binding domain of E1 is required to stimulate E2-dependent transcription, as all of the mutants that fail to bind DNA alone and in cooperation with E2 are also defective in this assay. Interestingly, LPM5 binds weakly to DNA on its own, but E2 can rescue its function in cooperative DNA-binding assays *in vitro* as well as rescue its replication function *in vivo* (Thorner *et al.,* 1993). These data also demonstrate that the carboxy-terminal domains of E1 important for helicase and replication functions are also required for such stimulatory activity.

E1 stimulation of transcription shows specificity for the E2 transactivation domain

Several studies have demonstrated that the BPV-1 E2 activation domain is important for interacting with E1 and that such interactions were important for replication (Lim *et al.,* 1998; Winokur and McBride, 1992). We were interested in determining whether the E1 stimulation of E2 enhancer function required the E2 activation domain. Alternatively, the E1 stimulation we observe could be due to its ability to recruit a cellular factor to a complex important for transcription. E1 has been shown to interact with DNA polymerase, and no evidence precludes its interaction with components of the general transcriptional machinery or with factors important for both replication and transcription.

We took advantage of the modular nature of the E2 transcription factor to address the role of specific E1:E2 interactions in the E1 stimulation of E2 enhancer function. We fused the E2 activation domain and hinge region (amino acids 1 to 325) to the amino-terminal DNA-binding domains of the tet repressor (tetR), to test the ability of this fusion protein to stimulate transcription and be activated by E1. We used a VP16–tetR fusion construct in comparison. In mammalian cell culture, eukaryotic activation domains, when fused to tetR, can activate transcription from promoters containing tet operator sites in a tetracycline-dependent manner (Gossen and Bujard, 1992).

Both the E2–tetR fusion and the VP16–tetR fusion were able to stimulate transcription from a reporter containing tet operator-binding sites, as measured by the luciferase activity. Expression vectors for each fusion protein were transfected into BEF cells with the tet-operator reporter construct as well as pCG-E1TTL as a negative control. After transfections, the cells were incubated in the presence or in the absence of tetracycline. The stimulation of luciferase activity detected in these assays was dependent on tet-repressor DNA binding, as the same constructs failed to stimulate transcription when tetracycline was present in the cell culture medium.

E1 stimulation of the activity of either of these fusion constructs in the presence or in the absence of tetracycline was tested. Cells were transfected as described above, with the addition of pCG-E1. As shown in Fig. 5A (black columns), E1 stimulated the enhancer function of the E2–tetR fusion protein, but not the VP16–tetR fusion. This E1 stimulation of E2 enhancer function was not

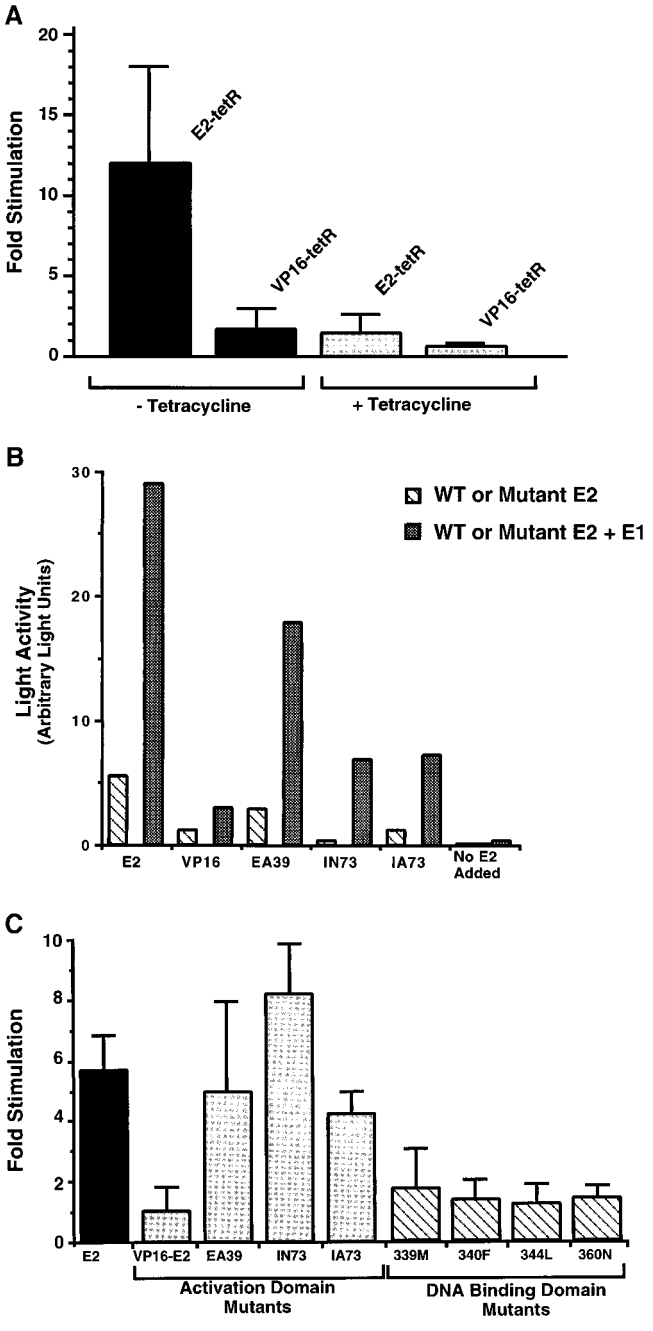

FIG. 5. E1 requires the E2 activation domain to stimulate enhancer function. (A) 50 ng of the E2-tetR and VP16-tetR activation domain fusion constructs was transfected into BEF cells with 250 ng of the tet-Luc reporter and 5 μg of pCG-E1 or pCG-E1TTL. Cells were transfected and incubated in the presence (+tetracycline) or in the absence (−tetracycline) of 1 μg tetracycline/ml in the cell culture medium. Fold effect of E1 on activation domain fusion constructs was then determined by dividing fusion + E1/fusion + E1TTL. These data are representative of six independent electroporations. Error bars represent 1 standard deviation. (B) 250 ng of the URR–Luc reporter was electroporated into cells with 50 ng of the wild-type or mutant pCG-E2 and 5 μg of pCG-E1 or E1 TTL. Luciferase activity was assayed in cell extracts harvested 2 days after transfection. Stimulation of reporter gene expression varied dramatically among the various E2 mutants transfected with E2 and the E1TTL control expression vector, consistent with previously characterized phenotypes. (C) The fold stimulation of mutant or wild-type E2 transactivation by E1 was determined by dividing light activity of E1 + E2 construct/E1TTL + E2 construct. Fold stimulation was an average of eight independent experiments.

seen when the E2–tetR fusion protein is unable to bind DNA (in the presence of tetracycline). These data suggest that direct interactions between the E2 activation domain and E1 are important for E1 stimulation of E2 enhancer function.

E1 rescues the enhancer function of a transcriptionally defective E2 mutant

Mutational analysis of the E2 activation domain and DNA-binding domain has been performed by several groups. Several point mutants in the E2 activation domain were made, some of which were found to genetically separate the functions of E2 (Abroi *et al.,* 1996; Brokaw *et al.,* 1996; Ferguson and Botchan, 1996; Grossel *et al.,* 1996); in one mutant the replication function was retained while transcription was reduced, and in another mutant the reverse was found. Mutants in the DNA-binding and dimerization domain of E2 have also been made. One of these mutants, W360N, is unable to dimerize and as a consequence fails to bind DNA. Other mutants in the DNA-binding domain, 339M, 340F, and 344L, are able to dimerize, but they bind DNA at significantly reduced levels (Prakash *et al.,* 1992); consequently, these mutants are impaired for their ability to stimulate transcription and replication (Li *et al.,* 1994; Prakash *et al.,* 1992). By using these point mutants we hoped to address whether the ability of E1 to stimulate E2 enhancer function correlated with the ability of E2 to stimulate replication and furthermore, to determine whether the cooperative interactions between E1 and E2 could rescue E2 point mutants.

As shown in Fig. 5C, E1 is unable to rescue mutants of E2 that are defective for DNA binding. These mutants show reduced transcription in the presence and in the absence of E1. A fusion construct, VP16–E2, that contains the VP16 activation domain fused to the E2 DNA-binding domain stimulated transcription; however, E1 had no effect on the enhancer function of this fusion protein (Figs. 5B and 5C). These data suggest that the primary effect of E1 on E2 enhancer function occurs through interactions with the E2 activation domain. Thus, although E2 DNA binding is necessary for this effect, a specific E2 DNA-binding domain is not required, since an E2–tetR fusion construct is also able to be stimulated by E1.

E1 could stimulate the enhancer function of several activation domain mutants. IN73 and IA73, two mutants that function as wild type in DNA replication but are defective for transcription, are actually rescued in their ability to function as transcriptional enhancers by E1.

Additionally, E1 could stimulate the activity of another E2 activation domain mutant, EA39. EA39 stimulation of transcription is similar to wild type; however, its ability to stimulate replication *in vivo* is impaired (Ferguson and Botchan, 1996). This result indicates that though E1 does not productively interact with EA39 for DNA replication, interactions for transcription can be indirectly assessed. Perhaps E1:E2 interactions for transcription are less stringent than are those required for DNA replication. Figure 5C shows a quantitation of the fold induction effected by E1 upon these mutants. We suggest that E1 may in some way modulate the structure of the E2 activation domain, perhaps stabilizing a particular conformation of E2 that enhances the factor's ability to act as an enhancer protein. Along these lines it is interesting to point out that many of the mutants in the E2 activation domain are temperature sensitive. IN73 and IA73, for example, are wild type in their transactivation function at reduced temperatures (Ferguson and Botchan, 1996). E1 may stabilize an active conformation for IN73 and IA73 in much the same way that reduced temperature seems to stabilize these proteins.

CONCLUSIONS

In this report, we demonstrate that the papillomavirus E1 protein plays a role in modulating the enhancer function of transactivator E2, *in vivo.* Other reports have similarly addressed the role of E1 in modulating E2 enhancer function. A key conclusion from our results is that reporter concentration influences the sign of this modulation. E1 stimulated E2-dependent transcription at low reporter concentrations, and this stimulation decreased with increasing concentrations of reporter, with E1 repression of transcription being observed at the highest reporter concentrations used. The repression by E1 of E2 function reported by Sandler *et al.* (1993) in BEF cells is consistent with our results at high reporter concentrations; however, the magnitude of repression was less in our data and we find no requirement for an E1-binding site for E2 stimulation. It is possible that other variations in the reporters, cell lines, and transfection conditions used contributed to these differences. Along these lines, we found that E1 had no effect upon E2 enhancer activity in C127 cells, though synergistic positive activity was indeed found in HeLa and C33A cells, while in CV-1 cells only repression was detected at the highest levels of reporter (Parker, 1996). The repression we see at high reporter concentrations is more consistent with the observations reported by Le Moal *et al.* (1994). In that report repression was ascribed to "squelching" caused by the extremely high levels of expression vectors and reporter plasmids transfected into the cells. Results of Ferran and McBride (1998), which show similar effects of E1 mutants that are compromised for DNA binding on repression of E2 enhancer function, may also be consistent with E1 repression caused by either squelching or partitioning of active E2 away from the reporter to the large amounts of bacterial plasmid carrier DNA with weak E2 sites (Lim *et al.,* 1998) used in their experiments. Thus according to our hypothesis, at low levels of reporter concentrations, E2 and E1 site concentrations are such that cooperative interactions between E1 and E2 allow for loading of a starting E2:E1 complex onto DNA. This complex then may allow for subsequent loading of multiple E2 molecules to tandem *cis*-acting E2 sites on the reporter. This in turn results in maximal transcriptional activity. At higher levels of *cis*-acting sites, one may suppose that E1 loads many E2 dimers to too many different molecules, leading to a situation wherein not enough E2 (or other *trans* activity factors) can build an effective enhanceseosome.

Though BPV-1 requires the E1 inverted repeats for effective DNA replication *in vivo,* certain data show that the E1 protein can productively interact with nonspecific DNA. Furthermore cooperative DNA binding between E1 and E2 for transcription may have a less stringent requirement for E1-binding sites than for *in vivo* replication complexes. E1 has been shown to unwind and replicate DNA in an *ori*-independent fashion *in vitro* (Fouts *et al.,* 1999; Yang *et al.,* 1993), and for BPV replication, *in vivo* strong E2-binding sites compensate for mutations in the *ori* E1-binding site (Sedman and Stenlund, 1995). Furthermore, mutational analysis of certain serotypes of HPV *ori* regions shows a stronger dependence on E2-binding sites for replication efficiency than E1, suggesting that the specificity of binding may be due to HPV E2 cooperatively binding E1 (Chiang *et al.,* 1992b; Remm *et al.,* 1992; Russell and Botchan, 1995). The requirements for the DNA-binding domains of E1 for E2 activation may thus be naturally expected for both nonspecific and specific protein DNA complexes mediated by this factor.

Cooperative interactions on DNA are likely to be important for E1's ability to stimulate E2 transactivation. It has been established that the BPV E1 protein interacts with full-length E2 and that these interactions are important for the replication of BPV *ori*-containing plasmids *in vivo* and *in vitro* (Li and Botchan, 1993, 1994; Lim *et al.,* 1998; Ustav and Stenlund, 1991; Yang *et al.,* 1991a). Based on our findings, it is reasonable to postulate that these interactions are similarly important for the effects of E1 on E2 enhancer function. However, our analysis of activation domain mutants of E2 in this assay leads us to suggest that cooperative DNA binding does not by itself explain E1's stimulation of E2 enhancer function. We found that E1 could rescue the enhancer function of E2 activation domain mutants defective for transcription *in vivo.* For example, the E2 mutant IN73 shows less than 1% of E2 activity, but in the presence of E1, transcription reporters yield activities close to wild-type E2 levels. This result indicates a function for E1 beyond a tethering activity, for IN73 binds to DNA with wild-type affinity.

Because E1 is unable to stimulate the enhancer function of a heterologous activation domain, we favor a model in which E1 enhances or stabilizes a transcriptionally "active" conformation of E2, which then could potentially allow more productive interactions with cellular factors important for transcription. However, our results do not rigorously prove that the effects of E1 are due to direct protein:protein interactions between E1 and E2. Indeed Demeret *et al.* (1998) have presented data that indicate that E1 may contain cryptic transcriptional activation regions and that these may be targeted to DNA or enhanced by E2. At present this later thought and other even more complicated models cannot be ruled out, but we note that Demeret *et al.* (1998) required multiple tandem *cis* elements to detect weak E1 transactivation and that in our data E1 by itself showed no transactivation. Thus our model can simply explain the current data with the viral URR.

In transcription complexes many proteins exert allosteric or stabilizing effects on other proteins that alter or enhance activity. For example, the transcription factor ATF-2 is thought to be activated by the adenovirus E1a through stabilization of a particular conformation. The bZIP domain of ATF-2 is thought to be masked *in vivo* through direct interaction between the bZIP domain and an N-terminal zinc finger (Abdel-Hafiz *et al.,* 1993). Association with E1a exposes this domain, allowing transcriptional activation.

Our data may also be consistent with the notion that E1 cooperative interactions with E2, including allosteric effects on the activation domain, may facilitate interactions between E2 and a cellular factor. Interactions with this cellular factor could be very weak in transcriptionally compromised E2 mutants, and E1 may be able to rescue these mutants by stimulating the interactions between E2 and a cellular factor. Both E1 and E2 interact with a variety of cellular replication and transcription factors (Benson *et al.,* 1997; Bonne-Andrea *et al.,* 1995; Cueille *et al.,* 1998; Li *et al.,* 1991; Park *et al.,* 1994; Rank and Lambert, 1995).

We suspect that one form of a DNA replication preinitiation complex involving E1 and E2 is responsible for the enhanced transcription activity detected. This because E2 has been found to leave such complexes in an ATP-dependent step prior to double hexamer formation and we assume that E2 must therefore function prior to its release (Lusky *et al.,* 1994; Sanders and Stenlund, 1998). In this regard it is curious that mutants in the E1 ATP-binding domain are defective in transactivation activity. Given that E2 is likely to interact with a rather large surface of E1 including regions in the DNA-binding domain and the carboxy-terminus of the helicase initiator, one might posit that ATP binding per se is not critical for transactivation but rather the native conformation of this domain is critical. E1 ATP binding and multimerization may thus be processes that compete with a stable E1:E2 complex in *cis* on the *ori* sites. The recent crystal structure of a human form of the E2 activation domain (Harris and Botchan, 1999) shows that the surface has multiple and scattered regions important for replication and transcription. Furthermore, flexible surface loops of E2 are found that might be expected to change conformation upon interaction with E1. It will clearly be important to obtain further mutational and structural analyses of the E2:E1 interaction domains to test the idea that E1 might transiently stabilize a transcriptionally active form of E2.

What roles may the enhancer-modulating activities of E1 play in the viral life cycle? Here, we are particularly intrigued by the possibility that P_{89} activity is enhanced by E1 protein and that this process is autocatalytic. After increased E1 levels occur, one may expect DNA amplification and subsequent repression of both the P_{89} and the P_{7940} promoters to manifest perhaps a down-modulation of the viral replicon by simple titration of the E2 protein by mechanisms discussed above.

MATERIALS AND METHODS

Plasmids

Wild-type and mutant BPV E1 and E2 proteins were expressed from plasmids containing the CMV promoter in the plasmid pCG-ATG$^-$ as described by Tanaka and Herr (1990). The pCG-E2 and pCG-Eag expression vectors, which make the BPV E2 and E1 gene products, respectively, have been previously described (Ustav and Stenlund, 1991), as have the expression vectors that make the E1 DNA-binding and ATP-binding mutants (MacPherson *et al.,* 1994; Thorner *et al.,* 1993), the E2 activation domain and DNA-binding domain mutants (Ferguson and Botchan, 1996; Prakash *et al.,* 1992), and the VP16–E2 and VP16–tetR fusion proteins (Gossen and Bujard, 1992; Li *et al.,* 1991). The E1TTL mutant pCG-E1TTL was constructed by inserting a translation termination linker into the *Sma*I site at E1 nucleotide 945.

E2–TetR fusion genes were made by cloning PCR products of E2 into pUHD14-1Sma (Baron *et al.,* 1997) encoding the Tet repressor (Baron *et al.,* 1997). PCR products were made using the primer E2TF5 at the 5′ end of E2 (5′CCC GAA TTC CCG GGG GGT GCC ATG GAG ACA GCA TGC GAA C) and the E2TF3L primer (5′CCC GGA TCC TTA TCT AGA CCC CCC TCC TGC CTT TAA CAG) at the 3′ end of E2 to encode the first 325 amino acids of E2. The 5′ primer has *Eco*RI and *Xma*I sites engineered at the 5′ end of the primer, and the 3′ primers contain engineered *Bam*HI and *Xba*I sites at the 5′ end of the primer. The E2 activation domain was cloned upstream of the Tet repressor DNA-binding domain using *Eco*RI and *Xba*I to generate fusion proteins. All fusion proteins are expressed from the CMV promoter.

The reporter plasmid pUHC13-3, containing tet operator sites upstream of the minimal CMV, has been de-

scribed previously (Gossen and Bujard, 1992). The *Eco*RI-*Bsa*I backbone fragment from the pUHC131-1 luciferase (Bonin *et al.,* 1994) and the *Eco*RI–*Bsa*I fragment from the URR containing pH100 (Lusky and Botchan, 1984) were fused together via ligation to create URR–Luc. A *Bgl*II–*Pfl*mI fragment from the pT81 luciferase containing plasmid (Nordeen, 1988) and a *Bgl*II–*Pfl*mI fragment from the E2RE1tk-CAT reporter were ligated together to create the E2RE1tk-Luc reporter. E2RE1tk-CAT was constructed through a ligation of a *Hin*dIII-blunted Nde1 vector fragment from the plasmid pBLCAT, derived from the −37tkCAT plasmid (Luckow and Schutz, 1987), to a *Sma*I–*Hin*dIII fragment of the fragment UR229. UR229 contains E2RE1 from nucleotides 7598 to 7831 cloned into the pUC18 polylinker through a sticky/blunt ligation of the *Bam*HI–*Bgl*II BPV fragment to the *Bam*HI–*Hin*cII sites in the pUC18 polylinker.

The mutant URR–Luc reporter plasmids were constructed using the following strategy. PCR products containing wild-type or mutant *oris* were engineered with the 5′ 7805 primer (5′-GCTCGAAACCGCCTTAAAC-3′) and the 3′ 22 primer (5′-CTCGAATTCAGGTCCATGTG-3′), which has an engineered *Eco*RI site. All mutant origins have been previously described (Mendoza *et al.,* 1995). The PCR products containing wild-type or mutant *oris* were cut with *Bgl*I and *Eco*RI and ligated to a *Mlu*I–*Bgl*I insert containing a wild-type or mutant E2RE1 region of the BPV genome and a *Mlu*I–*Eco*RI vector fragment of URR–Luc. All constructs were subsequently sequenced to confirm that mutant constructs had been obtained. Plasmid clones and PCR products were made using standard protocols (Sambrook *et al.,* 1989).

Cell culture

Primary BEFs, kindly provided by Barbara Spalholz (National Institutes of Health), were grown in 10-cm plates until 80% confluent. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, and 100 U/ml penicillin. Cells were trypsinized and diluted 1:5 to passage them. These cells were never used after passage 12. For studies using tet fusion constructs, inactivation was achieved by incubation of cells with cell culture medium containing 1 μg tetracycline per milliliter of medium, immediately posttransfection.

Transfections

Transfections were done using electroporation, essentially as previously described (Ustav and Stenlund, 1991). Briefly, cells were split 24 h prior to electroporation, and 2–5 × 10^6 actively growing cells were trypsinized and pooled. Cells were resuspended in 0.25 ml Dulbecco's modified Eagle's medium with 10% fetal calf serum and 5 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (pH 7.2). The DNA samples, as described in the figure legends, were mixed with 25 μg of sheared salmon sperm DNA and the 0.25-ml cell suspension. This mixture of cells and DNA was then transferred to an electroporation cuvette (0.4-cm gap Bio-Rad Gene Pulser Cuvettes). Cells were electroporated at 250 V at a capacitance of 960 μF with a Bio-Rad Gene Pulser. Cells were then removed from the cuvette and aliquotted into one or two 60-mm plates and harvested after 48 h. Due to the effects of E2 on a variety of heterologous promoters (Haugen *et al.,* 1988; Parker and Botchan, unpublished observations), no internal controls could be used for normalizations of transfection efficiencies. All experiments were done in duplicate and were repeated a number of times to ensure reproducibility.

Luciferase assays

Cells were harvested from 60-mm plates using 500 μl of a buffer containing 0.5% Tween 20, 2 mM CDTA, and 25 mM bicine, pH 7.8. Plates with the lysis buffer were frozen at −80°C for no less than 30 min. Plates were thawed and the lysates were used directly or refrozen at −80°C until assayed, and samples were spun in a microfuge for 10 s to bring down cellular debris. Luciferase assays were performed using the continuous count method outlined by Promega Technical Bulletin 480 (Promega, 1993). Essentially, 100 μl of the luciferase assay reagent containing 20 mM Tricine, pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, pH 7.8, 33.3 mM DTT, 270 μM Coenzyme A, 470 μM luciferin, 530 μM ATP, was mixed with 30 μl cell extract in a luminometer cuvette. Samples were measured in a Turner 2000 luminometer with a 5-s delay and a 15-s integration period, and the resulting readings were expressed as arbitrary light units.

RNA isolation and primer extension

BEF cells were transfected as described in the legend to Fig. 1C and RNA was isolated from six identically transfected 10-cm plates 48 h after transfection. Total RNA was isolated by the method of Chomczynski and Sacchi (1987), except samples were resuspended in 100 μl TE, pH 8.0, with no SDS added. The concentration of the RNA was measured using a spectrophotometer at an absorbance of 260 nm. Primer extension analysis was performed as described in *Current Protocols in Molecular Biology* (Ausubel *et al.,* 1987). Briefly, the oligo CAT-PE (5′ GCC ATT GGG ATA TAT CAA CGG TGG-3′) was labeled using standard kinase reaction conditions. The oligo was then annealed to 15 μg of *in vivo* isolated RNA. RNA and primers were incubated at 75°C for 2 min and incubated at 55°C for 1 h in a 10-μl volume of 10 mM Tris–Cl, pH 7.8, 1 mM EDTA, 1.25 M KCl. Annealing reactions were then mixed with 40 μl extension mix (62.5 mM Tris–Cl, pH 8.3, 12.5 mM DTT, 1.25 mM $MnCl_2$, 125 μg/ml actinomycin D, 0.33 mM dNTPs) and 1.0 μl (10 U)

murine leukemia virus reverse transcriptase (Promega), incubated at 37°C for 45 min and stopped with 300 μl 100% ethanol. DNA was precipitated, washed, and resuspended in formamide loading dye. Samples were denatured by boiling before they were separated by electrophoresis in parallel with a DNA sequence ladder and visualized by autoradiography.

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