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The bovine papillomavirus 1 E2 protein contains two activation domains: one that interacts with TBP and another that functions after TBP binding

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The E2 transactivator of bovine papillomavirus type-1 is unable to activate minimal promoters *in vivo* that contain only E2 binding sites and a TATA box. This block can be overcome by over-expression of human TATA binding protein (TBP) or by the addition of either SP1 binding sites or an initiator element to the promoter, suggesting that the binding of TFIID may normally be a rate-limiting step for activation by E2. Surprisingly, purified E2 and TBP bind co-operatively to DNA *in vitro* when the sites are closely spaced. E2 does not affect the on rate of association but reduces the off rate. The E2 region responsible for this effect is located in the hinge region that links the classic transactivation and DNA binding domains. We demonstrate that the TBP stabilizing domain contributes *in vivo* to co-operativity with co-expressed TBP and to activation of the major late minimal promoter (MLP) containing E2 sites. In contrast, promoters with SP1 sites are activated to wild-type levels by such a mutant. This promoter specificity is also evident *in vitro*. A truncated E2 mutant, lacking the classic transactivation domain but containing the TBP stabilizing domain, stimulates transcription of the MLP *in vitro*, but does not activate promoters with SP1 sites. In conclusion, our results show that the E2 transactivation domain has a modular structure. We have identified one domain which probably acts at an early step in the assembly of the pre-initiation complex and which is involved in reducing the dissociation rate of bound TBP *in vitro*. The classic N-terminal activation domain of E2 might affect one or several step(s) in the assembly of the pre-initiation complex occurring after the binding of TFIID.
Key words: BPV-1 E2/co-operativity/pre-initiation complex assembly/TBP/transactivator

Introduction

In eukaryotic RNA polymerase II-dependent promoters, multiple binding sites for one or more activators are usually located upstream of the core promoter elements such as the TATA box or initiator. One way in which activators might modulate the activity of a promoter would be to facilitate rate-limiting steps in the assembly of the pre-initiation complex (PIC). An activator might do this by directly contacting one or more of the general transcription factors, which might recruit them to the PIC or stabilize the complex after its assembly. Other possibilities would be that activators compete with the binding of factors that repress transcription, such as DR1, DR2 (Inostroza *et al.*, 1992), NC1 and NC2 (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991), or they might counteract repressive effects of chromatin (reviewed in Felsenfeld, 1992; Workman and Buchman, 1993).

In TATA box-containing promoters the initial step in the assembly of the PIC involves the binding of TFIID to the TATA sequence (reviewed in Roeder, 1991; Zawel and Reinberg, 1992). TFIID is a multisubunit complex composed of the TATA binding protein (TBP) which specifically recognizes the TATA box sequence (reviewed in Hernandez, 1993; Rigby, 1993) and at least seven TBP-associated factors (TAFs) (Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; Zhou *et al.*, 1992; Hoey *et al.*, 1993). After binding of TFIID, TFIIA and TFIIB are recruited to the complex (Moldonado *et al.*, 1990) which is then joined by RNA polymerase II, associated with TFIIF, followed by TFIIE, TFIIJ and TFIIH (Zawel and Reinberg, 1992), to form a functional PIC.

TATA-less promoters usually contain an initiator (INR) element which overlaps the transcriptional start site. An INR binding protein or a component of the TFIID complex may bind first to the specific initiator sequence (Kaufmann and Smale, 1994; Martinez *et al.*, 1994; Purnell *et al.*, 1994; Verrijzer *et al.*, 1994) and replace the DNA binding function of TBP in nucleating assembly of the PIC (reviewed in Hernandez, 1993). However, a TFIID complex containing TBP is also essential for transcription of TATA-less promoters (Pugh and Tjian, 1991).

Early studies indicated that binding of TFIID might be altered by activators (reviewed in Ham *et al.*, 1992). Activation domains of several proteins have been shown to interact directly or indirectly with TBP *in vitro*. For example, those of VP16, E1A or p53 can directly contact TBP (Stringer *et al.*, 1990; Horikoshi *et al.*, 1991; Lee *et al.*, 1991; Seto *et al.*, 1992; Chen *et al.*, 1993; Liu *et al.*, 1993; Martin *et al.*, 1993; Ragimov *et al.*, 1993; Truant *et al.*, 1993). Mutations in VP16 and E1A which reduce the interaction *in vitro* also reduce activation *in vivo* (Ingles *et al.*, 1991; Lee *et al.*, 1991), strongly suggesting that these interactions play a role in activation. However, it is still not understood how the binding of an activator to TBP might facilitate transcription. For example, in the case of E1A or VP16 it is not clear whether they recruit TBP to the TATA box or stabilize its binding. In *in vitro* transcription systems, TBP on its own can direct basal level transcription but the TFIID complex is necessary to mediate the stimulatory effects of all activators tested

until now (Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; Zhou *et al.*, 1992), even in the case of those which directly contact TBP (Boyer and Berk, 1993; Brou *et al.*, 1993; Lieberman and Berk, 1994). This suggests that the TAFs might also be a target of activation domains. Indeed, in the case of SP1 a direct interaction with TAF 110 has been demonstrated, and in a reconstituted *in vitro* transcription system it was found that these interactions are important for the stimulatory activity of SP1 (Hoey *et al.*, 1993; Weinzierl *et al.*, 1993). Furthermore, a single transcription factor may interact with several components and affect PIC assembly at several levels. Kinetic experiments have suggested that acidic activators like VP16 probably act after the binding of TFIID by accelerating a step involving TFIIA (Wang *et al.*, 1992; White *et al.*, 1992). In addition, *in vitro*, VP16 was shown to contact TBP and TAF 40 (Goodrich *et al.*, 1993) and to recruit TFIIB to the D–A complex (Lin and Green, 1991; Choy and Green, 1993). It is not clear at the moment whether all the protein–protein interactions demonstrated in *in vitro* systems are crucial for transactivation *in vivo*, or even whether they could occur at the same time or just transiently.

We are investigating how the E2 transactivator protein of papillomaviruses activates transcription and are attempting to identify its cellular targets. Like other transcription factors, the E2 protein has a modular structure with a transactivation domain rich in acidic amino acids and glutamines at its N-terminus, and with a C-terminal DNA binding and dimerization domain (reviewed in Ham *et al.*, 1991a; McBride *et al.*, 1991; Steger *et al.*, 1993). The bovine papillomavirus type-1 (BPV-1) E2 stimulates the activity of several viral promoters by binding to its sites which are present in multiple copies in the viral genome. It can also activate transcription from heterologous promoters when its binding sites are cloned either upstream or downstream of the transcription initiation site (Thierry *et al.*, 1990). However, in contrast to complex promoters, E2 is unable to stimulate minimal promoters containing only a TATA box and E2 sites (Ham *et al.*, 1991b).

In addition to its function as an activator, E2 can also act as a repressor, the precise position of its binding sites determining the nature of the effect. For example, in HPV (human papillomavirus)-18, E2 down-regulates the activity of the promoter at position 105, called P_{105}, which directs the expression of the viral oncogenes E6 and E7 by binding to a site located 3 bp upstream of the TATA box (Thierry and Yaniv, 1987; Thierry and Howley, 1991). We have shown that binding of BPV-1 E2 to this site interferes with the formation of the PIC probably by inhibiting TBP binding. However, by increasing the distance between the E2 sites and the TATA box to 8 bp, we observed that E2 and TBP bound co-operatively to this DNA in gel shift assays (Dostatni *et al.*, 1991). To test if a construct having such a spacing between the E2 sites and the TATA box would be stimulated by E2 *in vivo*, a promoter containing four E2 sites 8 bp upstream of the TATA box was constructed and tested in transfection experiments. To our surprise we found that E2 on its own was unable to activate this promoter, but over-expression of human TBP at the same time as E2 overcame this block with the two proteins activating transcription synergistically. These results suggested that binding of TFIID might be a rate-limiting step for activation by E2, even though E2 and TBP bind co-operatively to DNA *in vitro*. This conclusion was supported further by the observation that promoters containing, in addition to the TATA box, either the adenovirus major late promoter (MLP) INR sequence, SP1 sites or even TATA-less promoters with the INR and two SP1 sites, could be activated very efficiently by E2 on its own (Ham *et al.*, 1994). SP1 and TFII-I, the cellular protein which binds to the MLP INR sequence, have both been shown to physically interact with TBP and other components of the TFIID complex (Hoey *et al.*, 1993; Roy *et al.*, 1993; Emili *et al.*, 1994). Since either over-expression of TBP or the presence of either SP1 sites or the INR element increases activation by E2, we concluded that the principal role of the E2 transactivation domain might be to affect a step in the formation of the PIC that occurs after the binding of TFIID. However, this stands in contrast to our findings *in vitro* that E2 and TBP bind co-operatively to DNA. To try to clarify this dichotomy between the *in vivo* and *in vitro* results, we have further characterized the interactions between E2 and TBP and have analyzed the functional relevance of the co-operative binding for activation by E2 *in vivo*.

Results

E2 and TBP bind co-operatively to DNA

To understand better the basis for co-operativity in DNA binding between E2 and TBP, we expressed the human TBP as a His-tagged fusion protein in bacteria and purified it to homogeneity. The E2 protein was expressed in yeast and purified from whole-cell extracts with the use of a heparin column followed by an E2-specific oligonucleotide affinity column (Dostatni *et al.*, 1991). As shown in Figure 1A, TBP binds to the DNA probe containing the P_{105} TATA box and one E2 site separated by 8 bp. The binding of the E2 protein gave rise to two bands in a gel shift. As the protein only shows one band in SDS–PAGE after gold staining and the ratio of the two bands varied depending on the temperature of the binding reaction (data not shown), we conclude that the two bands represent two conformations of the protein bound to DNA and do not result from proteolytic degradation.

Adding E2 and TBP together to the DNA probe leads to the appearance of a new complex, which migrates slightly faster than the TBP but slower than the E2–DNA complex alone (Figure 1A). This complex contains both proteins, as suggested previously by adding anti-E2 antibodies (Dostatni *et al.*, 1991) and by homologous competition. As demonstrated in Figure 1B, the TBP–E2 complex is competed by adding a molar excess of a TATA box containing oligonucleotide to the reaction. Furthermore, the addition of an oligonucleotide containing an E2 site results in loss of the co-operativity, demonstrating that the co-operativity between E2 and TBP is dependent on the binding of E2 to its site. Finally, the complex is largely unaffected by a non-specific competitor (an SP1 site). Quantitation of the gel in Figure 1A using a PhosphorImager showed that the binding of E2 and TBP to the DNA probe containing one E2 site and a TATA box, separated by eight nucleotides, is strongly co-operative. For example, at the lowest concentration used in the experiment in Figure 1A, TBP only bound 3% of the

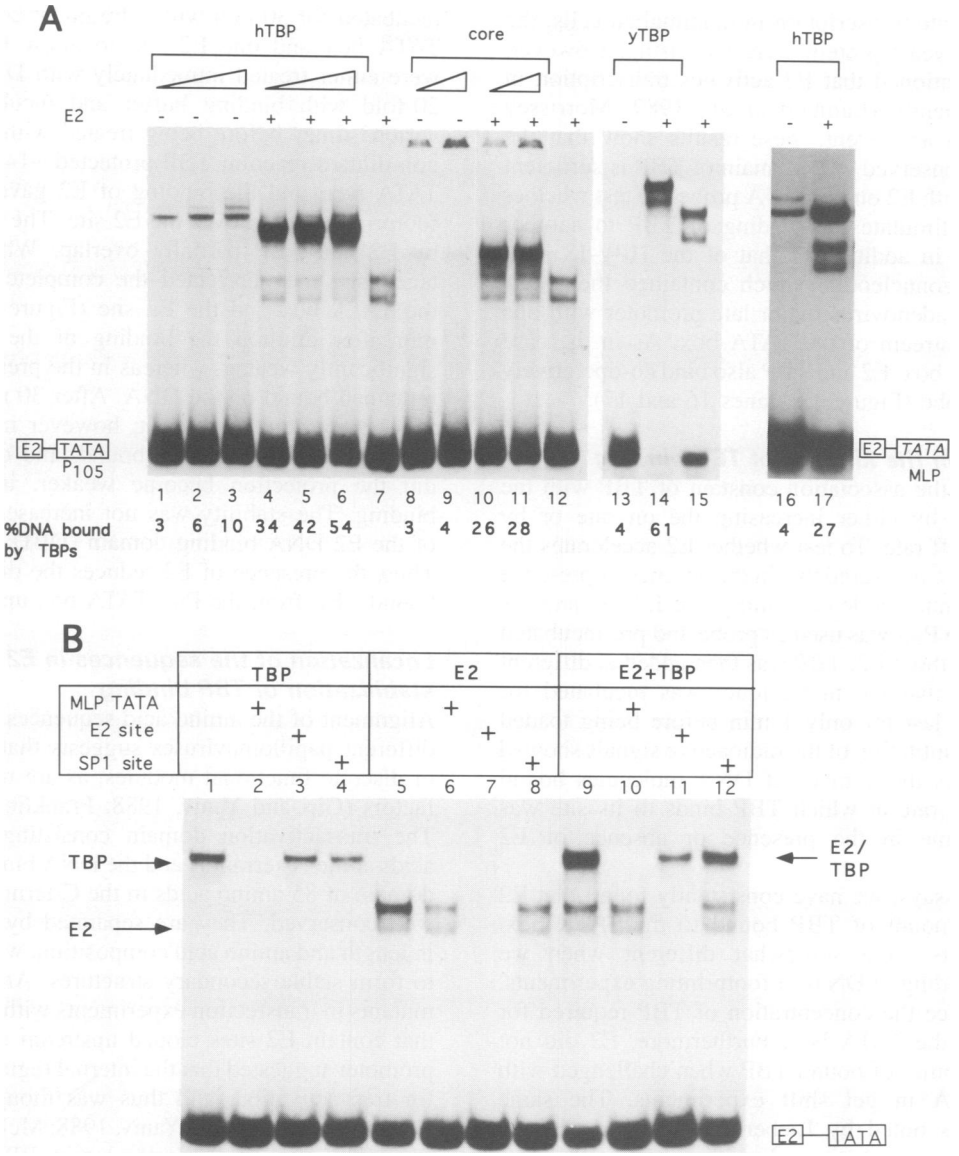

Fig. 1. E2 and TBP bind co-operatively to DNA. (**A**) Purified E2 and human TBP (hTBP), the 180 C-terminal conserved amino acids (core) and yeast TBP (yTBP), and 2 fmol of a labeled oligonucleotide containing one E2 site and the TATA box of the HPV-18 P_{105} promoter (lanes 1–15) or one E2 site and the TATA box of the adenovirus MLP separated by eight bases (lanes 16 and 17), were incubated and analyzed on a 5% native polyacrylamide gel. 5 (lanes 1 and 4), 10 (lanes 2 and 5) and 20 ng (lanes 3 and 6) of TBP or 10 (lanes 8 and 10) and 20 ng (lanes 8 and 11) of the core protein were incubated either alone (lanes 1–3, 8 and 9) or together (lanes 4–6, 10 and 11) with 0.1 ng of E2 as indicated above the lanes. The binding of 0.1 ng E2 alone is also shown in lane 12. In the case of the yTBP, 0.35 ng of E2 and 10 ng of yTBP were incubated either alone (lanes 13 and 15) or together (lane 14) with the probe before loading onto the gel. The radioactive signals were quantitated with the use of a PhosphorImager and the percentage of the probe, which was bound by TBP, is indicated below the lanes. (**B**) E2 and TBP bind specifically to their sites. The probe containing one E2 site and the P_{105} TATA box separated by eight nucleotides was incubated with either TBP alone (lanes 1–4), with E2 alone (lanes 5–8) or with E2 and TBP together (lanes 9–12). Competition experiments were performed by adding excess unlabeled oligonucleotides containing the TATA box of the MLP (lanes 2, 6 and 10), an E2 site (lanes 3, 7 and 11) and an SP1 site (4, 8 and 12). The positions of the complexes are indicated.

probe; in the presence of E2 the fraction of DNA molecules to which TBP had bound had increased to 34%. Conversely the binding of E2 is also stimulated by TBP (data not shown).

TBP is composed of an evolutionarily conserved C-terminal domain, also called the core domain, and an N-terminal region which varies in length and sequence (Hoffmann *et al.*, 1990). The 180 amino acid C-terminal core domain of TBP is sufficient for DNA binding and basal transcription, and can be assembled into the TFIID complex (Zhou *et al.*, 1993). We have shown previously in transient transfection assays that the core domain of human TBP is sufficient for co-operation with E2 in activation of the P_{105} minimal promoter *in vivo*, while the yeast (y) TBP could not substitute for the human protein (Ham *et al.*, 1994). When tested in our *in vitro* gel shift assay both the core of human (h) TBP and the full-length yTBP could co-operate with E2 in *in vitro* DNA binding. As shown in Figure 1A, the human (h)core and the yTBP alone gave rise to weak bands only, while in the presence of E2 the binding of both the hcore and the yTBP was strongly stimulated. Since E2 and the yTBP do not co-

operatively activate transcription in mammalian cells, the results with the yeast protein were surprising. However, it should be mentioned that E2 activates transcription in yeast very efficiently (Lambert *et al.*, 1989; Morrissey *et al.*, 1989). In any event, these results show that the evolutionarily conserved core domain of TBP is sufficient for interaction with E2 on this DNA probe. To test whether E2 could also stimulate the binding of TBP to another TATA sequence in addition to that of the HPV-18 P_{105}, we used an oligonucleotide which contained the TATA sequence of the adenovirus major late promoter with one E2 site 8 bp upstream of the TATA box. As in the case of the P_{105} TATA box, E2 and TBP also bind co-operatively to this DNA probe (Figure 1A, lanes 16 and 17).

Effects of E2 on the kinetics of TBP binding

E2 could affect the association constant of TBP with the P_{105} TATA box by either increasing the on rate or by decreasing the off rate. To test whether E2 accelerates the rate of binding we measured this in the absence or presence of E2. An oligonucleotide containing one E2 site and the TATA box of the P_{105} was used as probe and pre-incubated either with or without E2. TBP was then added at different time points, so that the first aliquot was incubated for 30 min and the last for only 1 min before being loaded onto the gel. Quantitation of the radioactive signals showed that E2 increases the number of DNA molecules bound by TBP but the rate at which TBP binds to its site was roughly the same in the presence or absence of E2 (Figure 2A).

In gel shift assays, we have consistently found that E2 increases the amount of TBP bound to the TATA box. However, results were somewhat different when we studied TBP binding in DNase I footprinting experiments. E2 did not reduce the concentration of TBP required for full binding to the TATA box. Furthermore, E2 did not increase the stability of bound TBP when challenged with competitor DNA in gel shift experiments. The same discrepancy was noted by Lieberman and Berk (1991) when they were analyzing the co-operative binding between the Epstein–Barr virus (EBV) activator protein Zta and TBP.

In studying the kinetics of TBP binding, Hoopes *et al.* (1992) suggested that the binding of TBP might occur in a two-step pathway. The first step might involve an equilibrium association between the DNA and protein to give a metastable but specific complex which is then converted into the final stable complex, detectable in a gel shift assay. This final step may involve an isomerization event of TBP (Hoopes *et al.*, 1992). In gel shifts, the reaction mixture is diluted when loaded onto the gel. According to the model of Hoopes *et al.* (1992), this dilution might result in dissociation and separation of non-stably bound molecules from the reaction. In footprinting experiments, on the other hand, the binding is assayed in the concentrated reaction and might not discriminate between stably bound and metastably bound forms. To test whether E2 might increase the amount of TBP stably bound to the TATA box, we performed footprint experiments where we diluted the reaction before treatment with DNase I and analyzed the binding of TBP in the presence or absence of E2, an approach similar to that used by Lieberman and Berk (1991). E2 and TBP were incubated for 30 min with a fragment containing the P_{105} TATA box and one E2 site to allow binding. Samples were either treated immediately with DNase I or diluted 20-fold with binding buffer and incubated further for various times before being treated with DNase I. In the non-diluted reaction TBP protected ~14 bp over the P_{105} TATA box, and the binding of E2 gave rise to a 17 bp footprint centered over the E2 site. The regions protected by E2 and TBP partially overlap. When added simultaneously, they protected the complete region spanning the TATA box and the E2 site (Figure 2B). Already 10 min after dilution the binding of the TBP alone was significantly weaker, whereas in the presence of E2, TBP remained bound on the DNA. After 30 min, TBP binding was nearly completely lost; however in the presence of E2, TBP remained stably bound, and only after 60 min did the protection become weaker, indicating loss of binding. The stability was not increased in the presence of the E2 DNA binding domain (DBD; data not shown). Thus, the presence of E2 reduces the dissociation rate of bound TBP from the P_{105} TATA box upon dilution.

Localization of the sequences in E2 responsible for stabilization of TBP binding

Alignment of the amino acid sequences of E2 proteins of different papillomaviruses suggests that E2 is composed of discrete functional modules, as are many transcription factors (Giri and Yaniv, 1988; Franklin and Kim, 1991). The transactivation domain consisting of ~200 amino acids at the N-terminus and the DNA binding/dimerization domain of 85 amino acids in the C-terminus are relatively well conserved. They are separated by a region varying in length and amino acid composition, with low propensity to form stable secondary structures. Analysis of deletion mutants in transfection experiments with reporter plasmids that contain E2 sites cloned upstream of the SV40 early promoter suggested that the internal region is not necessary for transactivation, and thus was thought to serve as a flexible linker (Giri and Yaniv, 1988; McBride *et al.*, 1989). In addition to the full-length protein, BPV-1 expresses two repressor forms (called E2TR and E8/E2) lacking most or all of the transactivation domain (reviewed in Steger *et al.*, 1993). To localize the region in E2 which mediates the co-operative binding with TBP, we expressed and purified different deletion mutants of E2 and tested them in gel shift assays with TBP. As shown in Figure 3A, E2TR, which starts at amino acid 161, and $E2_{\Delta 1-203}$, which corresponds to the E2 segment present in the E8/E2 repressor form, both stimulated the binding of TBP to at least the same extent as the full-length E2 (Figure 3A, lanes 7–9). In contrast, the DBD (amino acids 325–410) only increased the binding of TBP between 1.0- and 1.5-fold (Figure 3A, lanes 11 and 12). These results suggest that the region in E2 involved in co-operative interaction with TBP is not localized within the N-terminal transactivation or the C-terminal DNA binding/dimerization domains, but rather within the non-conserved middle part of E2. Furthermore, the mutant with an internal deletion from amino acids 158 to 282 ($E2_{\Delta 158-282}$) only marginally stimulated TBP binding (Figure 3A, lane 10), like the DBD which maps the active element to amino acids 204–282. To try to confirm this, we introduced further internal deletions in the E2TR protein which can still co-operate

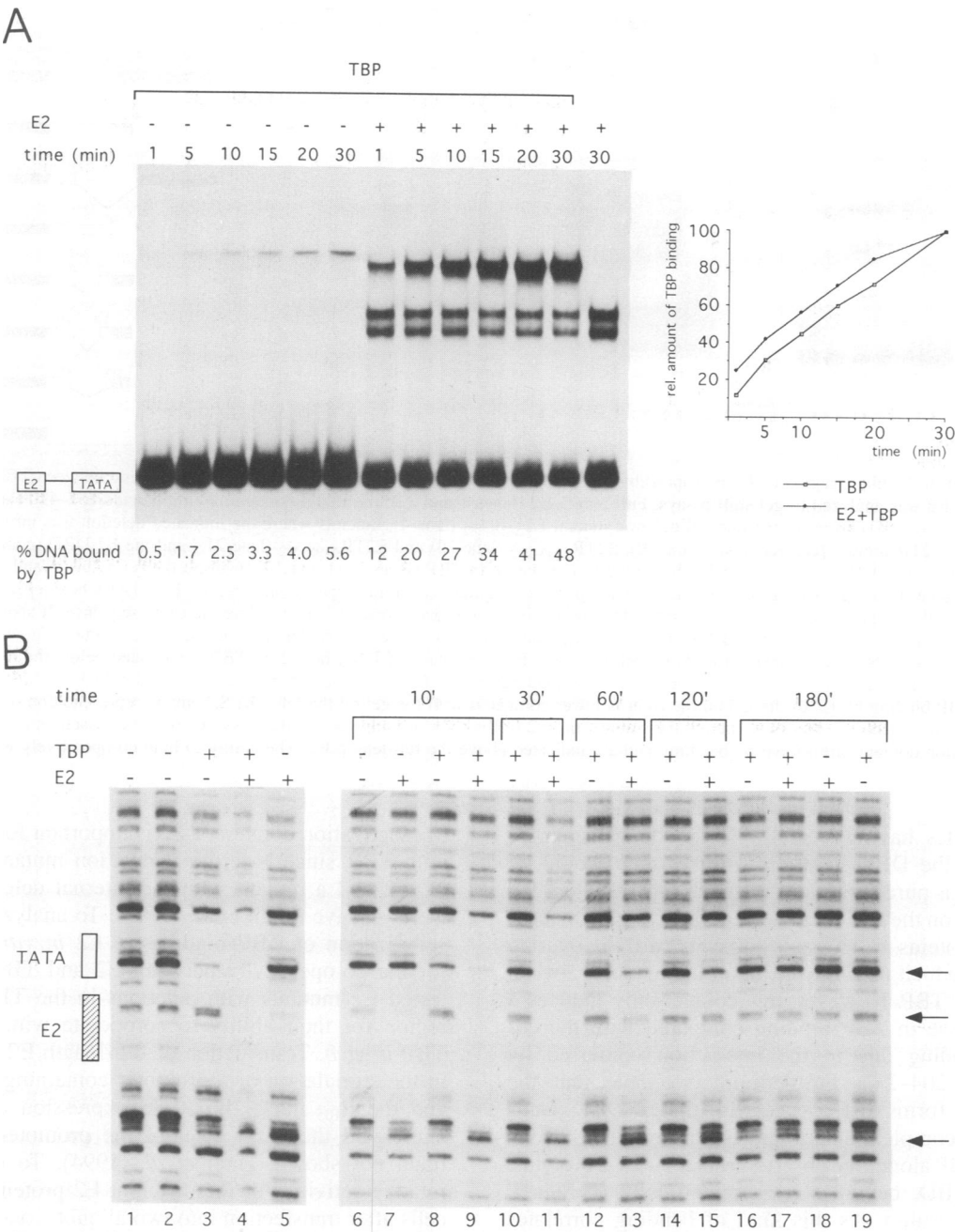

Fig. 2. E2 does not accelerate the rate at which TBP binds to DNA but stabilizes it upon dilution. (**A**) 50 pg of an oligonucleotide containing one E2 site and the P_{105} TATA box was either incubated with 5 ng hTBP alone or together with 0.2 ng of E2 for 1, 5, 10, 15, 20 or 30 min as indicated in the figure before being loaded onto the gel. The gel was scanned by a PhosphorImager and the percentage of DNA bound by TBP is given below the respective lanes. The graph shows the percentage of TBP binding during 30 min. The total amount of TBP–DNA complex after 30 min either with E2 (◆) or without E2 (◇) was set at 100%. The percentage of TBP bound at the different time points was calculated and plotted against time. The slopes of the two curves are parallel, indicating that the kinetics of TBP binding are not changed in the presence of E2. (**B**) DNase I protection of the P_{105} TATA box and one E2 site, separated by 8 bp, in the presence of E2 and TBP. In lanes 1, 2 and 6 no proteins were added. E2 alone (lanes 5, 7 and 18), TBP alone (lanes 3, 8, 10, 12, 14, 16 and 19) or both proteins together (lanes 4, 9, 11, 13, 15 and 17) were incubated for 30 min to bind. The samples in lanes 1–5 were treated with DNase I immediately. Samples in lanes 6–19 were diluted 20-fold and incubated further as indicated in the figure before being digested with DNase I. The positions of the TATA box and the E2 site are indicated on the left. The arrows indicate the most important bands which are protected in the presence of the proteins; the lowest arrow points to a strong hypersensitive site which is induced by the binding of E2.

with TBP. Figure 3A (lanes 13–15) shows that $E2TR_{\Delta 195-282}$, $E2TR_{\Delta 213-282}$ and $E2TR_{\Delta 220-282}$ had a lower capacity to co-operate with TBP than the full-length E2 protein. There might be a gradual decrease in co-operativity with increasing the deletion, as the co-operativity of the $E2TR_{\Delta 195-282}$ was reduced compared with the other two mutants, as observed in several experiments (data not shown). Taken together, these results suggest that sequences between positions 204 and 282 provide the major motif for the co-operative interaction, but amino acids between 161 and 195 may also contribute to the interaction with TBP. In addition, the fraction of

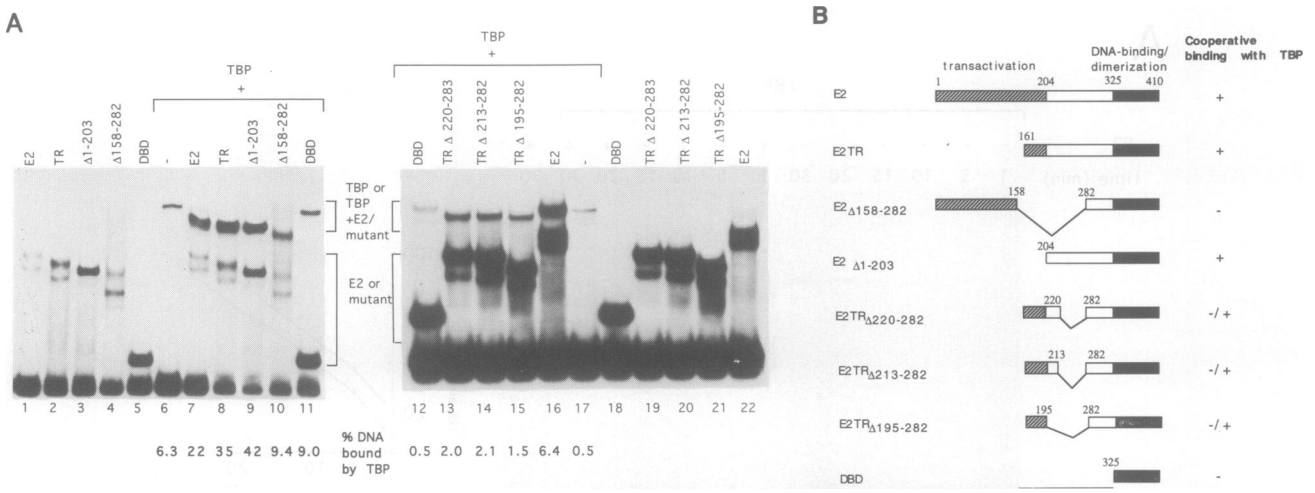

Fig. 3. A segment in the hinge region of E2 is responsible for co-operative binding with TBP. (**A**) Co-operative binding between TBP and different purified forms of E2 was analyzed in gel shift assays. Full-length E2 (lanes 1 and 22), the E2TR containing amino acids 161–410 (lane 2), $E2_{\Delta 1-203}$ starting with amino acid 204 (lane 3), the internal deletion mutant $E2_{\Delta 158-282}$ (lane 4), mutants where the indicated deletion was introduced within the context of the E2TR protein [$E2TR_{\Delta 220-282}$ (lane 19), $E2TR_{\Delta 213-282}$ (lane 20) and $E2TR_{\Delta 195-282}$ (lane 21)] and the E2 DBD (consisting of amino acids 325–410; lanes 5 and 18) were incubated either alone or together with TBP (lanes 7–11 and 12–16 respectively). Lanes 6 and 17 show the binding of TBP alone. In the gel shown on the left, a labeled purified fragment containing one E2 site and the P_{105} TATA box separated by eight nucleotides was used as a probe; in the gel on the right, the probe was the oligonucleotide $E2_8T$ encoding the same sequence. It should be noted that the double complex, consisting of E2 and TBP bound to the fragment (lane 7), migrated faster than that bound to the shorter oligonucleotide (lane 16). The radioactive signals were scanned on a PhosphorImager and the percentage of DNA bound by TBP is indicated below the respective lanes. As the binding of TBP on its own is already higher in the right-hand gel (6.3%, lane 7) compared with the left-hand gel (0.5%, lane 17), the fold stimulation of TBP binding by E2 in the gel on the right is lower compared with the gel on the left. (**B**) Schematic representation of E2 and the different E2-derived mutants used in (A). The numbers above the E2 ORFs refer to amino acids; the positions of the transactivation and the DNA binding/dimerization domains are shown by hatching and are indicated above the full-length E2. The ability to bind co-operatively with TBP is indicated.

DNA molecules having bound TBP in the presence of $E2_{\Delta 158-282}$ or the DBD is slightly higher than would be predicted for a purely random distribution. E2 and TBP are very close on the DNA and indeed the regions protected by the two proteins in DNase I footprints partially overlap (Figure 2B), which might result in an interaction between the DBD and TBP. However, this contact only induces a modest increase in TBP binding compared with the co-operative binding due to the interaction conferred by amino acids 204–282. Furthermore, we note that the binding of all forms of E2, except for the E2 DBD, leads to a double complex that migrates faster than the one containing TBP alone. We observed that all the E2 proteins, except the DBD, bend DNA asymmetrically (G.Steger, unpublished results). As this kind of bending correlates with an increased mobility of the ternary complex containing E2, TBP and the DNA, we suppose that this increased mobility might be due to DNA bending induced by E2 binding to its site.

Figure 3B shows a schematic representation of the different E2 mutants and their ability to stimulate TBP binding. The segment of E2 most crucial for the co-operativity was localized to the internal region from amino acids 204 to 282, and is not part of the transactivation domain mapped previously.

The region of E2 responsible for TBP stabilization in vitro contributes to co-operativity with TBP in vivo

As mentioned above, core promoters which contain only a TATA box and E2 sites are weakly activated by E2 on its own, but E2 strongly co-operates with over-expressed TBP in activation. We have shown previously that the transactivation domain of E2 is important for co-operation with TBP, since N-terminal deletion mutants Δ1–15 and Δ1–52 and a mutant with the internal deletion Δ92–161 were inactive (Ham *et al.*, 1994). To analyze whether the stabilization of TBP binding by E2 *in vitro* is essential for the co-operativity between E2 and TBP *in vivo*, we tested E2 mutants with deletions in the TBP interacting region for their ability to co-operate with co-expressed TBP *in vivo*. Transfection of full-length E2 did not result in the stimulation of a promoter containing four E2 sites and the P_{105} TATA box, and expression of TBP alone stimulated the basal level of the promoter 3- to 5-fold (data not shown; Ham *et al.*, 1994). To determine the relative activities of the different E2 proteins we divided cells after transfection into two aliquots: one was used for the chloramphenicol acetyl transferase (CAT) assay and another for the preparation of nuclear extracts. We co-transfected 0.5 μg of TBP and 100 ng of the expression vectors for each different E2 protein, an amount of plasmid which is not sufficient to reach maximal activity and does not result in squelching with any of the proteins, as determined previously. In the experiment shown in Figure 4, the P_{105} promoter was stimulated 105-fold in the presence of TBP and E2 (Figure 4A, lane 1). Co-expression of TBP and $E2_{\Delta 195-282}$ still stimulated the activity of $p4E2T_{105}$ 71-fold. $E2_{\Delta 220-282}$ increased the promoter's activity 19-fold and $E2_{\Delta 1-203}$ 21-fold in co-operation with TBP (see Figure 4, lanes 2, 3 and 5). Since the mutant proteins accumulated to different levels in the transfected cells, we determined the concentration of the different forms of E2 by performing gel shifts with 20 μg of nuclear extract and an E2 binding site as probe, and quantified the DNA binding activities. Figure 4B shows that

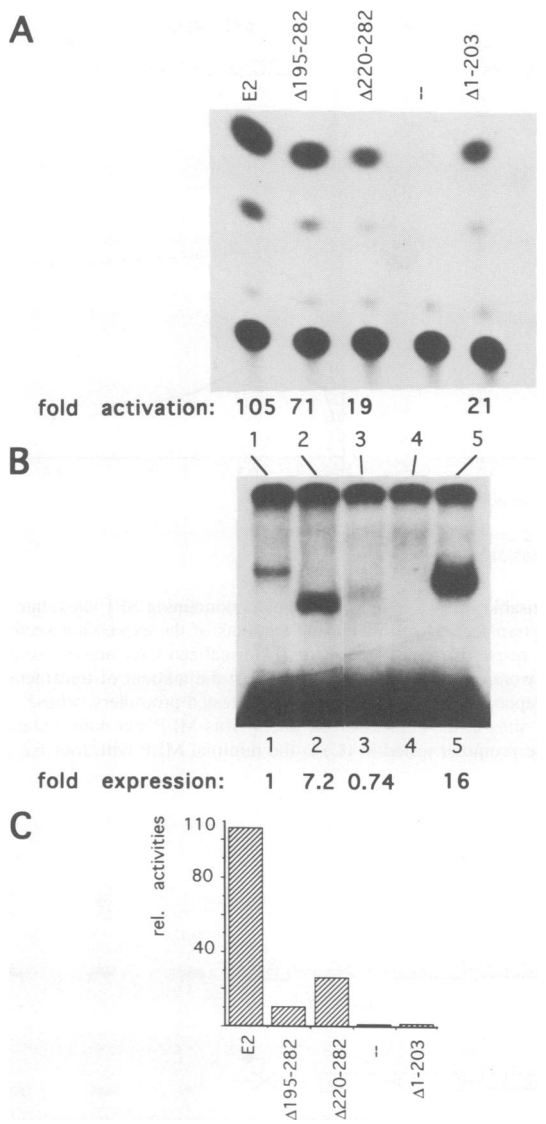

Fig. 4. Co-operativity between E2 and TBP *in vivo* requires the domain in E2 which was shown to stabilize the binding of TBP *in vitro*. Either 100 ng of expression vector for E2 (lane 1), for $E2_{\Delta 195-282}$ (lane 2), for $E2_{\Delta 220-282}$ (lane 3), for $E2_{\Delta 1-203}$ (lane 5) or as control the empty expression vector containing only the SV40 early promoter (lane 4), were transfected into RAC 65 cells together with $p4E2T_{105}$ as reporter plasmid and 0.5 μg of the expression vector for hTBP. (**A**) Normalized CAT activities were determined 48 h after transfection and the fold activation is shown at the bottom of the respective lanes. (**B**) Gel shift performed with 20 μg of nuclear extract, prepared from another aliquot, and an E2 site containing oligonucleotide. After scanning, the E2-specific signal was set as 1. The fold expression of the mutants compared with full-length E2 is given under the respective lanes. (**C**) Diagram of the relative activities of the different forms of E2 in the activation of $p4E2T_{105}$ with co-expressed TBP.

$E2_{\Delta 195-282}$ and $E2_{\Delta 1-203}$ accumulated to 4.7- and 16-fold higher levels than the intact protein. In contrast, $E2_{\Delta 220-282}$ was expressed to a lower level than E2. We then divided the CAT activities observed in the presence of limiting amounts of transactivators by the E2 concentrations. The values obtained from this normalization show that $E2_{\Delta 195-282}$, which only poorly increased the binding of TBP *in vitro*, stimulated the promoter 9.8-fold. The deletion mutant $E2_{\Delta 220-282}$, retaining slightly higher co-operativity *in vitro*, increased the activity of the promoter 25.6-fold *in vivo* compared with 105-fold by the wild-type protein. In contrast, the relative activity of the $E2_{\Delta 1-203}$, which contains the entire TBP interacting region, is only 1.3-fold higher (Figure 4C). This confirms that in equimolar concentrations the N-terminal activation domain of E2 is required for E2 to efficiently co-operate with co-expressed TBP *in vivo*. In addition, the region in E2 which stabilizes the binding of TBP *in vitro* is necessary for full activation. However, the TBP interacting domain on its own can mediate co-operativity with co-expressed TBP when present in high amounts, as seen in the co-transfection experiment in Figure 4A (lane 5).

The hinge segment is necessary for full activation of the adenovirus MLP, but is dispensable for a promoter containing SP1 sites

We have shown previously that promoters containing SP1 sites or an INR element can be activated by E2 on its own, suggesting that on these promoters the binding of TFIID may no longer be rate limiting (Ham *et al.*, 1994). To analyze the role of the TBP stabilizing segment of E2 in activation of these types of promoter, we compared the transactivation potential of wild-type E2, which strongly co-operates with TBP, with that of $E2_{\Delta 195-282}$, which only poorly co-operates with TBP in *in vitro* binding. We transfected increasing amounts of expression vector for E2 or for $E2_{\Delta 195-282}$ with the CAT reporter plasmid to measure the maximal activities of the wild-type protein and the mutant. First, we tested two promoters which contain four E2 sites upstream of two SP1 sites in the context of the adenovirus MLP, but with either the TATA box or the INR element mutated (p4E2Smli and p4E2Smlt). The TATA box-containing promoter was stimulated 61-fold by the full-length E2, as seen in Figure 5B. The deletion mutant could activate this promoter 50-fold. The SP1 site containing TATA-less promoter (p4E2Smli) is stimulated 10-fold by the wild-type protein, whereas the mutant protein reaches a higher value which was 13-fold stimulation (Figure 5A). Previously, we found that E2 could also strongly activate the minimal adenovirus MLP consisting of the TATA box and an initiator element when four E2 sites have been cloned upstream. As described before, we find that E2 can also increase the binding of TBP to the TATA box on the MLP promoter. To test whether the activation involves the TBP interacting region in E2, we also compared the activity of the deletion mutant with that of E2 on this promoter. Figure 5C shows that with 500 ng of E2 expression vector, a maximal stimulation of 48-fold is obtained. Only a 21-fold increase in the activity is reached by transfecting 500 ng of $E2_{\Delta 195-282}$ expression vector and a further increase in its concentration results in a squelching effect.

These results suggest that the TBP stabilizing segment in E2 is largely dispensable for activation of promoters containing SP1 sites. In contrast, it significantly contributes to maximal activation of the minimal MLP.

E2, starting at amino acid 204, stimulates the activity of the MLP in vitro, but not a SP1 site containing promoter

The *in vitro* gel shift assay and the transfection studies *in vivo* revealed the existence of a TBP stabilizing region

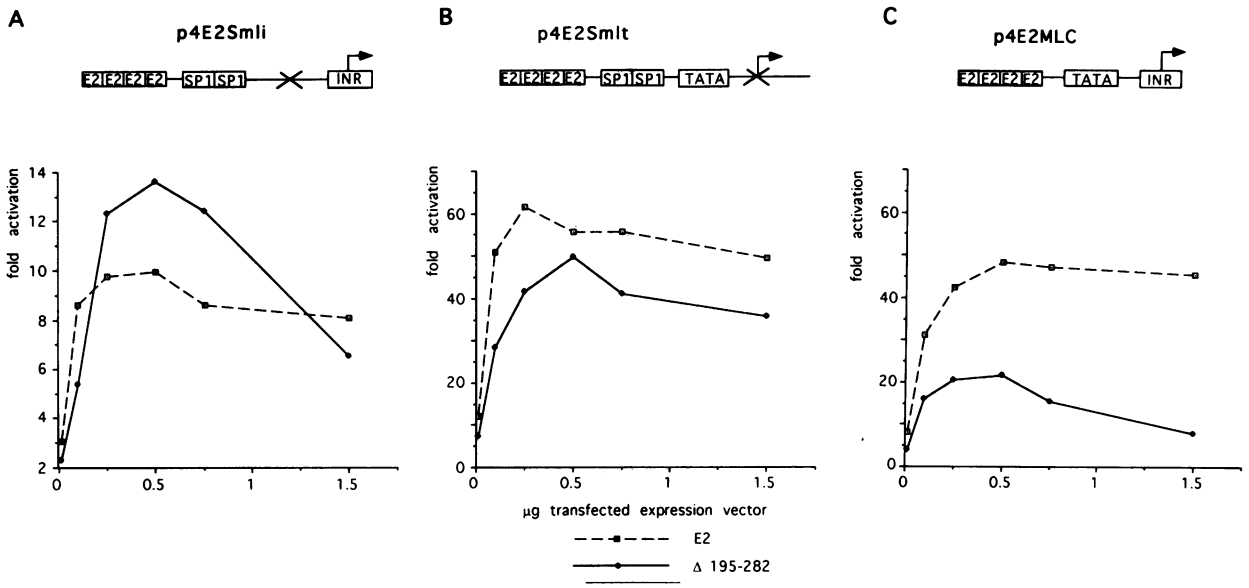

Fig. 5. Amino acids 195–282, which include the TBP stabilizing segment, are dispensable for activation of promoters containing SP1 sites, but contribute to maximal activation of the adenovirus minimal MLP. Rac 65 cells were transfected with increasing amounts of the expression vector for E2 or the deletion mutant $E2_{\Delta 195-282}$ (Δ195–282), as indicated, and with 2 µg of the respective reporter construct. Normalized CAT activity was determined as described in Ham *et al.* (1994). Fold activations by E2 or the mutant were calculated and plotted against the amount of transfected expression vector. Each point represents the mean of the results obtained in four independent transfection assays. The tested promoters, whose structures are indicated above the panels, contain four E2 sites upstream of two SP1 sites in the context of the adenovirus MLP, but with mutations within either the TATA box (p4E2Smli) (**A**) or the INR element (p4E2Smlt) (**B**). The promoter tested in (**C**) is the minimal MLP with four E2 sites (p4E2MLC).

located in the previously defined hinge segment of the E2 proteins. To determine if this region by itself can stimulate transcription, we tested the activity of purified $E2_{\Delta 1-203}$ on the different promoters in *in vitro* transcription using HeLa nuclear extracts as a source of general transcription factors. CAT reporter plasmids, described in the previous section, were linearized with *Eco*RI and used as templates in run-off transcription. As shown in Figure 6, $E2_{\Delta 1-203}$ is able to stimulate the MLP ~5-fold, as determined by scanning the gel with a PhosphorImager, whereas the activity of the promoter is not affected by adding the same molar amount of the DBD. The SP1 site containing promoter p4E2Smli, whose activation *in vivo* by E2 did not depend on this internal region, was not stimulated *in vitro* by either $E2_{\Delta 1-203}$ or the DBD. $E2_{\Delta 1-203}$ only marginally stimulated (1.3-fold) a promoter containing two SP1 sites and a TATA box with the INR mutated. $E2_{\Delta 1-203}$ was also able to stimulate the MLP promoter in transient transfection experiments 3-fold (data not shown). These results correlate with the observations found in transfection experiments with $E2_{\Delta 195-282}$, which suggests that part of the hinge region of the E2 of BPV-1 participates in activation of the MLP but is largely dispensable in activation of a promoter with SP1 sites. In addition, it demonstrates that the hinge region of E2 is able to act as an activation domain on its own.

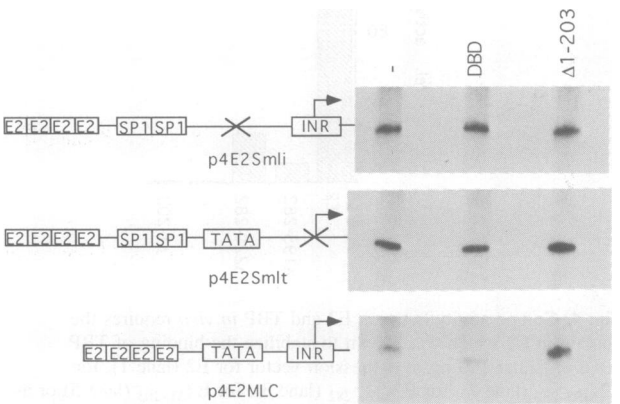

Fig. 6. The E2 mutant, starting at amino acid 203, can activate the MLP, but not the SP1 site containing promoters *in vitro*. Linearized CAT reporter plasmids were used as template in run-off transcription with HeLa nuclear extracts. Transcription from 100 ng of p4E2Smlt, 200 ng of p4E2Smli and 300 ng of pMLC was analyzed in the presence of purified E2 DBD, which represents amino acids 325–410, or of $E2_{\Delta 1-203}$ (Δ1–203), starting at amino acid 204, or with no protein added (−). The structures of the respective promoters are shown to the left of the respective signals.

Discussion

E2 stabilizes the binding of TBP to the TATA box in vitro

We have been studying the mechanism by which the BPV-1 E2 protein activates transcription and are trying to define the cellular targets of the E2 activation domain. In gel shift assays we found that purified E2 and TBP bind co-operatively to DNA probes when the E2 site and the TATA box are separated by eight nucleotides. Increases in the spacing between the two sites did not reveal any phasing phenomenon; however, the co-operativity decreased with increasing distance between the two sites (data not shown). The use of purified recombinant proteins excludes that the co-operation is mediated by a third factor, and strongly suggests that TBP might be a target of E2. Kinetic experiments showed that E2 reduces the

dissociation rate of TBP from the TATA box after dilution. We suppose that this might occur by a direct interaction between the two proteins on DNA, although we were unable to detect a strong specific interaction between E2 and TBP in solution by several techniques such as affinity columns, far Western blots or co-immunoprecipitations. It is possible that the interaction between E2 and TBP in solution is weak and below our detection level, while E2 and TBP interact strongly only when bound to DNA. Such a strong interaction might be possible if, for example, TBP underwent a conformational change upon binding to DNA, exposing a surface which can be stably bound by E2. There is evidence that TBP does indeed change its conformation by binding. For example, the binding of yTBP to the TATA box results in a change in its sensitivity to proteases (Lieberman *et al.*, 1991) and comparison of the crystal structure of free or DNA-bound TBP2 of *Arabidopsis thaliana* demonstrated that the protein undergoes a conformational change upon binding to DNA (Nikolov *et al.*, 1992; Kim *et al.*, 1993). In addition, by studying the kinetics of TBP binding, Hoopes *et al.* (1992) suggested that the stable binding of TBP might involve an isomerization step. Although we favor such a model, we still cannot totally exclude the possibility that E2 might enhance TBP binding by inducing structural changes on the DNA. We did not find, however, a correlation between the degree to which E2 deletion mutants bend DNA and their ability to stabilize TBP binding (unpublished observations).

Mapping the region responsible for stabilization of TBP binding revealed that it is outside the classic transactivation domain which was mapped previously by testing in-frame E2 deletion mutants for their ability to activate an SV40 promoter containing E2 sites or the long control region of BPV-1 (McBride *et al.*, 1989; Spalholz *et al.*, 1991). The internal domain of E2, which is responsible for stabilizing the binding of TBP, does not show any primary homology with known transcription factors.

Co-operative transcriptional activation in vivo and in vitro binding are related

Previous transfection experiments suggested that E2 might be unable to efficiently recruit TFIID to the TATA box and that it could act at an ulterior step (Ham *et al.*, 1994). The fact that E2 does not accelerate the on rate of TBP binding *in vitro* but stabilizes it on DNA is in agreement with this hypothesis. We also found that E2 co-operates with the core part of TBP both *in vivo* and in DNA binding. In contrast, although E2 increases the binding of yTBP *in vitro*, it did not co-operate in transfections. The core domains of yTBP and human TBP are 81% identical at the amino acid level (Hoffmann *et al.*, 1990). Since E2 can enhance the binding of both proteins *in vitro*, it presumably does so by contacting conserved residues. The inability of yTBP to mediate transcriptional activation by the retinoic acid receptor in Rac 65 cells, the same cells we used, has been described previously (Berkenstam *et al.*, 1992). This defect was found to be due to a failure of yTBP to interact with an E1A-like activity present in embryonic carcinoma cells (Keaveney *et al.*, 1993). It is likely that the inability of yTBP to mediate activation by E2 might be due to the same defect.

We demonstrate that in addition to the N-terminal transactivation domain, another region that we mapped in *in vitro* binding studies is necessary for maximal activity of the E2 protein. The TBP stabilizing domain by itself is not sufficient for strong activation *in vivo*, as the E2 protein lacking the N-terminal activation domain Δ1–203 only weakly co-operates with co-expressed TBP in activation of the $p4E2T_{105}$ promoter when present at equal concentrations to the full-length E2 protein. In high concentrations, however, the internal domain can mediate some co-operativity *in vivo* (Figure 4A, lane 5).

However, there are also several differences between the *in vitro* binding studies and the transcriptional data. Firstly, the co-operativity seen *in vitro* is the same in the presence of one or two E2 sites (data not shown), whereas *in vivo* activation increases with increases in the number of E2 sites (Ham *et al.*, 1994). E2 dimers may help each other to bind to DNA but, in the case of two strong E2 sites which we used *in vitro* and *in vivo*, this co-operativity is very low (Gauthier *et al.*, 1991; unpublished observations). A similar observation was made with VP16 which, in addition to TBP, contacts TAF 40 (Goodrich *et al.*, 1993) and TFIIB (Lin and Green, 1991). VP16 does not increase the amount of TBP bound, but it recruits TFIIB to the promoter. This recruitment was also possible in the presence of a single activator and did not change significantly in the presence of multiple activators which are necessary to activate transcription *in vivo*. Multiple activators acted on a later step *in vitro*, since they induced synergistic recruitment of general transcription factors which enter after TFIIB into the PIC (Choy and Green, 1993).

Furthermore, increases in the distance between the TATA box and the E2 site result in a decrease in co-operativity in DNA binding, whereas *in vivo* the co-operativity increases. One explanation for this discrepancy might be that *in vitro* we have only been looking at the E2–TBP contact, which on its own might not be strong enough to loop out intervening DNA and might only be energetically possible when both binding sites are very close. As discussed, complete activation will involve other domains in E2 performing additional contacts which might also be co-operative and thus be operating over a larger distance. Taken together, our results suggest that the interaction between E2 and TBP observed *in vitro* might contribute to the co-operativity *in vivo*, but could only be one of several steps required for activation.

The TBP interacting domain is required for maximal activation of the MLP, but is dispensable in the presence of binding sites for SP1

By analyzing different types of promoter, we found previously that E2 can co-operate with SP1 or the INR element of the MLP (Ham *et al.*, 1994). We find that the contribution of the TBP interacting domain in activation *in vivo* is different. We have confirmed these results by *in vitro* transcription with a mutant E2 form lacking the N-terminal transactivation domain but containing the hinge region including the TBP interacting part. What might happen on the different types of promoter?

The maximal activity of the deletion mutant $E2_{\Delta 195-282}$ is slightly reduced compared with the wild-type protein when the promoter contains a TATA box (Figure 5B), which might underline a role for the deleted region in the

binding of TBP, because the initiation pathway of the two types of promoter might differ in this step. In TATA box-containing promoters the initial step in the assembly of the PIC is the binding of TFIID, whereas in TATA-less promoters a TFIID complex containing TBP is also required, but it seems that on these promoters the DNA binding function of TBP is not involved (Pugh and Tjian, 1991; Martinez *et al.*, 1994). The deletion mutant activates a TATA-less promoter containing SP1 sites slightly better than wild-type E2 (Figure 5A), proving that despite the deletion the protein is functionally active. There are two possible explanations: (i) the TBP interacting domain may not be required for maximal activation in the presence of SP1 binding sites because the domain might not be able to contact its target in the presence of SP1; or (ii) the function provided by the E2-specific domain might be fully replaced by SP1 and thus be dispensable for activation. However, the functional redundancy by SP1 would be only part of the SP1 mechanism, as it is able to activate a promoter containing a TATA box on its own (Schmidt *et al.*, 1989; Hoey *et al.*, 1993; unpublished observations) whereas E2 cannot (Ham *et al.*, 1991b, 1994). It has been shown that transcriptional activation by SP1 involves interaction with TFIID. *In vitro*, SP1 was also recently shown to interact with TBP in *in vitro* binding studies (Emili *et al.*, 1994). Additionally, it contacts one of the TAFs, as shown for the *Drosophila* factors, and this is necessary for transcriptional stimulation by SP1 (Hoey *et al.*, 1993; Weinzierl *et al.*, 1993). In TATA-less promoters, which contain in addition to an INR element SP1 binding sites, an interaction of SP1 with TFIID or via a tethering factor, as suggested by Pugh and Tjian (1990), may help to recruit TFIID to the promoter (Pugh and Tjian, 1991). Thus, it might be possible that on both types of promoter the interaction of SP1 with TFIID might include the same function as is provided by the internal E2 domain.

On the MLP, the TBP interacting domain of E2 significantly contributes to activation (Figure 5C). The findings demonstrate that an N-terminally truncated mutant $E2_{\Delta 1-203}$ is able to activate the MLP *in vitro* (Figure 6). In addition, *in vivo* transfection assays (data not shown) show that this E2 domain, which includes the TBP interacting region, is able to function as an activation domain on its own. E2 can stimulate this promoter due to the presence of the INR element. An INR element was shown to co-operate with a TATA element in modulating the strength of a promoter, which might occur by enhancing the binding of TFIID through direct specific DNA binding by one of the TAFs to the INR element (Martinez *et al.*, 1994; Verrijzer *et al.*, 1994) or via an INR binding protein. One of the INR binding proteins, TFII-I, which was shown to bind to the MLP INR, can facilitate functional recruitment of TBP to the adenovirus MLP core promoter *in vitro* (Roy *et al.*, 1991, 1993). These observations and our binding studies suggest that the internal E2 activation domain and TFII-I might contact the same target, namely TBP. Additionally, these interactions would co-operate in activation.

The internal domain is also present in the two repressor forms of E2, which are expressed in BPV-1-infected cells. The fact that the E2 repressor forms are able to activate core promoters in contrast to complex promoters raises the possibility that they might play a role in the fine regulation of BPV-1-specific gene expression.

In conclusion, our functional studies show that the activation domain of E2 has a modular structure with distinct independently acting domains which might co-operate with different activators or basal factors to affect sequential steps in the assembly of the PIC. The classic N-terminal transactivation domain, which is conserved between the E2 proteins of different papillomaviruses, co-operates with SP1 probably by affecting a step in the assembly of the PIC which occurs after the binding of TFIID. In addition, the BPV-1 E2 has a domain which can function on its own as an activation domain and which contributes to activation by the full-length protein. The internal domain might act by affecting early steps in PIC assembly. It is unable to co-operate with SP1 (it seems likely that it might provide a function similar to that of SP1) but can co-operate with an INR element. We have identified this second activation domain by localizing the region of E2 which is responsible for co-operative binding with TBP *in vitro*, strongly suggesting (i) that this contact might be part of the mechanism by which the internal domain of E2 contributes to transcriptional activation and (ii) that stabilization of TBP binding might contribute to transcriptional activation. Furthermore, our functional study demonstrates that the role of an interaction, which may be revealed by *in vitro* assays and which can be correlated with a biological function, depends on the promoter context.

Materials and methods

Construction of plasmids

The yeast expression vector pPD2 and the construction of the pPD2 E2 expression plasmid, which was used for the production of full-length E2, are described in Lambert *et al.* (1989). For expression of the E2TR, an *Nco*I–*Hin*dIII fragment, starting with the original ATG of the repressor, was derived from the plasmid $pTZE2_{m\text{-}HdIII}$ (McBride *et al.*, 1988, 1989) and cloned together with a linker oligonucleotide containing *Hin*dIII–*Nco*I ends into the *Hin*dIII site of pPD2. The mutant $E2_{\Delta 158-282}$ was constructed by ligating the *Sph*I–*Hin*dIII fragment which was isolated from the plasmid $pTZE2_{\Delta 158-282}$ (McBride *et al.*, 1989) and thus already contained the deletion, with a linker oligonucleotide with *Hin*dIII–*Sph*I ends encoding the first five amino acids of E2 into the *Hin*dIII site of pPD2. For the construction of pPD2 $E2_{\Delta 1-203}$, a *Hin*dIII–*Fok*I oligonucleotide encoding an ATG and amino acids 204–211 of E2 was ligated to the *Fok*I–*Hin*dIII fragment of the E2 open reading frame (ORF) and cloned into pPD2. The expression vectors for $E2TR_{\Delta 195-282}$, $_{\Delta 213-282}$ and $_{\Delta 220-282}$ were constructed by isolating a *Nco*I–*Bam*HI fragment from the respective eukaryotic expression vectors used in Figure 4 (*Nco*I represents the ATG of the E2TR; described in McBride *et al.*, 1989); they were cloned into pET14B which was digested with *Nco*I–*Bam*HI. Standard procedures were used for cloning (Sambrook *et al.*, 1989). The sequences of the constructs, where oligonucleotides were used, were verified by direct sequencing of plasmid DNA using a USB Sequenase version 2.0 kit.

The E2 expression vector pC59 and the E2 deletion mutants $E2_{\Delta 195-282}$ and $E2_{\Delta 220-282}$ used in transfection experiments are described in McBride *et al.* (1989). The $E2_{\Delta 1-203}$ was obtained by subcloning the respective *Hin*dIII–*Bam*HI fragment from the yeast expression vector $pPD2E2_{\Delta 1-203}$ into the *Hin*dIII–*Bam*HI-digested pJ3 which contains the SV40 early promoter. The eukaryotic expression vector for hTBP is described as phTFIID in Berkenstam *et al.* (1992). These proteins are all expressed under the control of the SV40 early promoter. The construction of the CAT reporter plasmids $p4E2T_{105}$, p4E2Smli and p4E2Smlt are described in Ham *et al.* (1994).

Expression and purification of proteins

E2, E2TR, $E2_{\Delta 1-203}$ and $E2_{\Delta 158-282}$ were expressed in yeast. The yeast strain BWG1-7a: *leu2.2-leu2-11; his4-his519; ade1-ade100; ura3-ura52*

(Guarante and Mason, 1983) was grown in W_0 minimal medium supplemented with the required metabolites. Transformations were performed as described (Ito *et al.*, 1983) and yeast cells were grown on W_0 agar plates. Large-scale production and purification of the E2 proteins was performed mainly as described by Dostatni *et al.* (1991). Briefly, 1.6 l of pre-culture in W_0 medium (high phosphate conditions) were grown at 30°C to an OD_{600} of 2.0. After washing with sterile water, the cells were seeded in 4 l low phosphate synthetic medium with a starting OD_{600} of 0.6. They were grown for 12 h. A crude yeast extract was first loaded onto a heparin ultrogel column and the active fractions were purified further on an E2 site oligonucleotide affinity column. The proteins $E2TR_{\Delta 195-282}$, $E2TR_{\Delta 213-282}$ and $E2TR_{\Delta 220-282}$ were expressed in *E.coli* BL21pLys S and purified as described in Hoffmann and Roeder (1991), with the modification that the bacteria were lyzed in low salt buffer containing 100 mM NaCl. The proteins were purified as described for the other forms of E2. To assess their purity, the active fractions were loaded onto an SDS gel, blotted onto an NC-filter and stained with Proto-gold (Bio Cell Research Laboratories, Cardiff, UK). The proteins were found to be >95% pure. Their concentration was estimated to be 30 μg/ml, except for $E2_{\Delta 158-282}$ where the concentration was found to be 5 μg/ml. The purified E2 DNA binding domain was kindly provided by E.Androphy.

Human TBP was expressed in bacteria with a His-tag at the N-terminus of the protein and purified as described (Hoffmann *et al.*, 1990). As the hTBP was found to be only 50% pure after purification on an NTA–agarose column (Qiagen), the active fractions were pooled and purified further on an S-Sepharose Fast Flow (Pharmacia) column to >95% purity. The concentration of the hTBP preparation was estimated to be 0.5 mg/ml.

DNA binding assays

The double-stranded oligonucleotide $E2_8T$, containing the HPV-18 P_{105} TATA box and one E2 site separated by eight nucleotides, was used as a probe in most of the gel shift assays. It has the following sequence: 5′-TCGA**ACCGAAAACGGT**GATCCGTA**TATAAAAG**ATGTGT-3′ (the E2 site and the TATA box are in bold). The oligonucleotide, which contains the MLP TATA box and one E2 site, has the following sequence: 5′-TCGA**ACCGAAAACGGT**AGGGGGC**TATAAAAG**GGGGT-3′. The probes were labeled with the use of Sequenase in the presence of [α-^{32}P]GTP. For gel shifts, the binding reactions were carried out in a total volume of 20 μl in the presence of 12 mM HEPES (pH 7.9), 10% glycerol, 0.5 mM EDTA, 5 mM $MgCl_2$, 60 mM KCl, 4 mM spermidine, 0.1% NP-40, 500 ng bovine serum albumin (BSA), 250 ng poly d(G–C), 2 fmol probe and the purified proteins as indicated in the figures. The binding reactions were incubated for 30 min at 30°C and then loaded onto a low ionic strength 5% acrylamide gel (29:1) in 0.5× TBE buffer. The radioactive signals were quantitated on a PhosphorImager and the percentage of the probes bound by TBP calculated. For gel shift with nuclear extracts of transfected cells, 20 μg of nuclear extracts were pre-incubated in binding buffer with 1 μg poly(dIdC) and 1 μg of salmon sperm DNA for 20 min at room temperature before adding the probe containing one E2 site (5′-CTAGACCGAAAACGGTG-3′) and further incubation for 10 min on ice. DNA–protein complexes were analyzed in native 5% polyacrylamide gels with 0.5× TBE. For DNase I footprints, the binding reaction was carried out in a total volume of 10 μl in the same buffer as described for the gel shift reaction and in the presence 2.5 ng TBP, 3 ng E2 and 6 fmol asymmetrically labeled fragment (20 000–30 000 c.p.m.) containing the same sequence as the oligonucleotide $E2_8T$. After 30 min, the reactions were either treated directly with 5 ng DNase I (Boehringer) for 60 s after adjusting them to 2.5 mM $CaCl_2$, or they were diluted with 200 μl binding buffer and incubated further as indicated in Figure 2B. The reactions were then made for 2.5 mM $CaCl_2$, treated with DNase I and stopped with 60 μl stop (300 mM EDTA, 3% SDS, 450 mg/ml tRNA). After phenol/chloroform extraction and precipitation, the DNAs were electrophoresed on a 6% sequencing gel.

Cell culture and transient transfections

RAC 65 cells are derived from the mouse P19 embryonal carcinoma cell line and were used for transfection experiments (Berkenstam *et al.*, 1992). Cell growth, procedures for transient transfections and CAT assays have been described previously (Ham *et al.*, 1994). In detail, for the experiment shown in Figure 4, the reaction was scaled up 3-fold and 6 μg CAT reporter plasmid, 3 μg RSV (Rous sarcoma virus) β-gal, 1.5 μg pSG5.hTBP and 0.3 μg expression vector for E2 or the mutants, as indicated in Figure 4, were used for transfection of cells in 10 cm Petri dishes. Cells were harvested after 48 h to perform CAT assays and to prepare nuclear extracts of transfected cells as described by Schreiber *et al.* (1989). The radioactive signals of the gel shift were scanned in a PhosphorImager. The signal obtained in the presence of E2 was set as 1 and the ratio for the other forms was calculated. The relative activities were determined by dividing the fold activation by the fold expression. When performing dose–reponse experiments (results shown in Figure 5), 6 cm dishes were transfected with 2 μg CAT reporter plasmid and 1 μg RSV β-gal; the amount of expression vector for full-length E2 or $E2_{\Delta 195-282}$, as indicated in the text, was complemented with the plasmid pSG5 to 1.5 μg to keep the total amount of transfected SV40 promoter constant. The values shown represent the average of the results of four or five independent transfection experiments.

Nuclear extracts and in vitro transcription

HeLa S3 cells were maintained in spinner cultures in DMEM medium with 7% fetal calf serum. Nuclear extracts were prepared from freshly harvested cells essentially as described in Dignam *et al.* (1983) with the modifications described in Wildeman *et al.* (1984). The extracts were dialyzed against buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), aliquoted and frozen in liquid nitrogen. *In vitro* transcriptions were carried out in a total volume of 20 μl in 12 mM HEPES, 12% glycerol, 0.3 mM DTT, 0.12 mM EDTA, 40 mM KCl and 7.5 mM $MgCl_2$. The linearized templates were pre-incubated with the proteins for 5 min on ice, before adding 4 μl of nuclear extract and an additional incubation of 10 min at room temperature. Transcription was then initiated by the addition of 0.6 mM each of GTP, CTP and ATP, 0.12 mM UTP and 0.75 μl [α-^{32}P]UTP (Amersham); it was continued for 50 min at 30°C. RNA was purified and analyzed on a 4% denaturing polyacrylamide gel.

Acknowledgements

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