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Dose-Dependent Regulation of the Early Promoter of Human Papillomavirus Type 18 by the Viral E2 Protein

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The activity of the E6/E7 promoter of genital human papillomaviruses (HPVs) is positively and negatively modulated by a complex interplay between a variety of cellular transcription factors and the virally encoded E2 protein. The long control region of genital HPVs contains four E2 binding sites in conserved positions, two of which are very close to the TATA box. Binding of E2 to these two sites has been shown to repress the promoter. To carefully analyze the effect of E2 on the activity of the early promoter P105 of HPV18, we used an in vitro transcription system, which allowed titration of the amount of E2 protein. We found that low amounts of HPV18 E2 stimulated the promoter, whereas increasing amounts resulted in promoter repression. When the affinity was analyzed, it became obvious that E2 bound with highest affinity to E2 binding site 4 (BS-4), located 500 bp upstream of the promoter. The promoter most proximal binding site (BS-1) was the weakest site. Transient transfection assays confirmed that small amounts of HPV type (HPV18) E2 and also of bovine papillomavirus type 1 (BPV1) E2 were able to activate the P105, which was dependent on an intact BS-4. The positive role of BS-4 was also obvious at higher E2 concentrations, since mutation of BS-4 enhanced repression. In contrast to HPV18 E2, BPV1 E2 bound better to BS-1 and, in correlation, was able to more strongly repress the P105 in vivo. Our results suggest a dose-dependent regulation of the HPV18 E6/E7 promoter by E2 due to variable occupancy of its binding sites, which have antagonizing effects on the activity of the E6/E7 promoter.

Most papillomavirus (PV) types induce benign epithelial or fibroepithelial tumors of the skin or mucosa in humans and a variety of animals. However, infections with certain human PV (HPV) types have a high risk of progressing to carcinomas; HPV type 16 (HPV16) and HPV18 belong to this group (33, 55). In premalignant and malignant genital tumors, the expression of the viral oncogenes E6 and E7 is regularly elevated (2, 34). This finding suggested that upregulation of the early viral proteins E6 and E7 is a major factor contributing to malignant progression. In the viral life cycle, it is thought that E6 and E7 may facilitate viral DNA replication in differentiated noncycling cells (6). PVs infect the basal layer of the squamous epithelium, but viral DNA replication occurs only in the terminally differentiated keratinocytes. To initiate viral DNA replication, PVs rely mostly on host factors (for a review, see reference 8). However, the differentiated cells of the skin have already exited the cell cycle and thus have ceased to express the cellular replication genes. PV must reactivate these genes. E6 and E7 might do this by complexing and inactivating two negative regulators of cell proliferation, p53 and Rb (reviewed in references 51, 52, and 55). The inactivation of the tumor suppressors p53 and Rb may also lead to transformation (11, 52). These observations indicate that it is crucial for the virus to strictly control E6 and E7 expression to levels which allow viral replication but do not lead to malignant progression.

The expression of E6 and E7 is directed by the E6/E7 promoter in genital HPVs, which is tightly controlled by a complex interplay of positively and negatively acting host transcription factors, whose binding sites are present in the long control region (LCR) (reviewed in reference 48). These activities can be further modulated by the virally encoded E2 protein. E2 regulates viral transcription through binding as a dimer (15, 27) to its specific recognition sequence, $ACCN_6GGT$, which occurs several times in PV genomes (reviewed in references 19, 28, and 37). In addition, E2 is also required for viral DNA replication (50, 54). Bovine papillomavirus type 1 (BPV1) contains 17 E2 binding sites, and BPV1 E2 was shown to strongly activate transcription of several BPV1 promoters but also to repress one promoter (38). In HPVs, the number and distribution of E2 sites are different and E2 is only a moderate activator or mainly downregulates viral gene expression. For example, the late promoter of HPV8, which infects the skin, is activated at low amounts of E2, and a repression of basal promoter activity occurs at high amounts of E2. The activation is due to binding to high-affinity sites. The repression is mediated by binding of E2 to a low-affinity binding site, which overlaps with binding sites for cellular transcription factors necessary for basal promoter activity (39, 40). Genital HPVs contain four E2 binding sites within the LCR. The positions of these sites are highly conserved, indicating a common theme of regulation of the activity of the E6/E7 promoter by E2 (30) (Fig. 1). The early promoter was shown to be repressed by E2, which requires in the case of the HPV18 early promoter only the TATA box most proximal E2 binding site, BS-1 (10), whereas two promoter proximal sites, BS-1 and BS-2, are involved in repression of promoter P97 in HPV16 (31, 42, 43). These two E2 binding sites are spaced either 1 or 3 bp from the recognition sequences for the cellular transcription factors SP1 and TATA-binding protein (TBP). It has been shown by in vitro assays for HPV11, -16, and -18 that binding of E2 to BS-1 and BS-2 competes with the assembly of preinitiation complex and the binding of SP1 (10, 12, 14, 42, 43). The E2 binding site flanking the 3′ part of the enhancer, BS-3, was found to be involved in viral DNA replication (9, 41). A function for the promoter most distal site BS-4 has not been described yet. However, the strong conservation of the position of this site among the genital HPVs (30) indicates that this site may also be important for regulation of viral gene expression.

In transient transfection assays in different cell types, the

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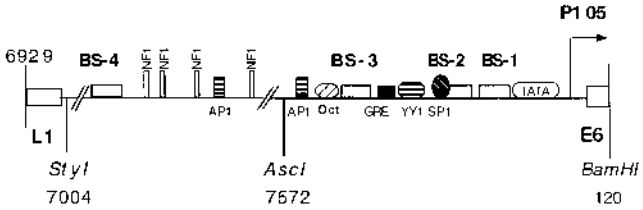

FIG. 1. Schematic representation of the LCR of HPV18. Binding sites for cellular transcription factors are indicated, as are the positions of the TATA box and the transcription initiation site of the P105 promoter. The binding sites for the viral E2 protein (BS-1, BS-2, BS-3, and BS-4) are also shown. The fragment spanning the region from positions 6929 to 120 was used in the different reporter constructs. The positions of the restriction enzymes which were used to isolate fragments for footprint experiments are indicated as well.

homologous full-length E2 protein seemed to repress weakly the early gene expression of genital HPVs (3, 7, 10, 12, 42, 46, 47), and even activation was reported (5). The BPV1 E2 protein consistently was a strong repressor, suggesting that BPV1 and HPV E2 proteins might functionally differ, although they share extensive homology in the DNA binding and transactivation domains (18). However, a recent study showed that E2 proteins of HPV16 and BPV1 are similarly strong activators when expressed in comparable amounts (49). The inconsistent results concerning the mode of action of E2 might in part be due to different levels of the various E2 proteins obtained in transfected cells. In addition, repression observed in transient transfection experiments might represent only the situation in the presence of relatively high E2 concentrations and bias E2 function toward repression. To systematically analyze a dose-dependent effect of E2 on the activity of the early HPV18 P105 promoter, we set up an in vitro transcription system with purified HPV18 E2 protein which allows us to exactly control the amount of E2. We demonstrate here that the HPV18 early promoter P105 is activated in the presence of low E2 amounts and repressed when the E2 concentration is increased. The promoter most distal, E2 BS-4, revealed the strongest affinity for E2. Binding of E2 to this site mediates a stimulatory transcriptional effect on P105 activity, which not only is obvious at low concentrations but also counteracts repression at higher E2 concentrations, as demonstrated by transient transfections.

MATERIALS AND METHODS

Cell culture and transient transfections. C33A cells were maintained in Dulbecco modified Eagle medium with Glutamax, supplemented with 7.5% fetal calf serum and the antibiotics streptomycin and penicillin. Transfection procedures have been described previously (20). In detail, 6-cm-diameter dishes were transfected with 2 or 3 μg (in the experiments shown in Fig. 6) of the luciferase reporter plasmid, 1 μg of pRSVβ-gal, and various amounts of expression vector, complemented to 250 ng with the vector pCMV1 in the case of HPV18 E2 and to 800 ng with pJ3, containing the simian virus 40 (SV40) early promoter in case of BPV1 E2, to keep the total amounts of transfected promoters constant. The cells were harvested 40 to 48 h after transfection and lysed in 100 mM KPO_4 buffer (pH 7.8)–1 mM dithiothreitol (DTT) by four freeze-thaw cycles prior to measurement of the luciferase activity in a Luminometer according to the manufacturer's instructions. To account for variations in transfection efficiencies, promoter activities were expressed as luciferase/β-galactosidase activity ratios, which were determined as described before (20). The values represent the averages of the results of four or five independent transfection experiments.

Construction of plasmids. The plasmids containing the HPV18 LCR (positions 6929 to 120) in front of chloramphenicol acetyltransferase (CAT) and pET14B-HPV18 E2 were kindly provided by Franciose Thierry and Caroline Demeret, Pasteur Institute, Paris, France, and have been described previously (10, 47). The eukaryotic expression vector for HPV18 E2 was constructed by cloning the *Asp*I-*Eco*RV (2783 to 4669) fragment of HPV18 into pCMV1 (38a). The expression vector for BPV1 E2 was pC59, which was described previously (54). 4321-Luc was constructed by cloning a *Hin*dIII-*Bam*HI fragment derived from the HPV18 LCR-CAT construct into the promoterless pALuc (13). Point mutations to mutate E2 binding sites present within the LCR were introduced into 4321-Luc by site-directed point mutagenesis using the PCR protocol as described in reference 1. The mutations introduced to abolish E2 binding contained the following sequences: BS-1, ACCGAAAACA**G**T; BS-2, ACCGAAA ACG**TG**; BS-3, ACCGAAATAG**CC**; and BS-4, ACCGATTTC**TA**T. The nucleotides in boldface represent the substituted bases. These mutations have been shown previously to abolish binding of E2 (26, 39, 47). All constructs have been verified by sequencing. Plasmid p4E2Smit has been described previously (20, 36).

Expression and purification of E2 of HPV18 and BPV1. HPV18 E2 protein was expressed in bacteria with a His tag at the N terminus of the protein and purified as described for human TBP (24). The protein was found to be 60% pure, and the concentration was estimated to 30 ng/μl by using a protein gel. To remove the His tag, the protein was first dialyzed against 150 mM KCl–20 mM Tris (pH 8.0)–10% glycerol–2.5 mM $CaCl_2$ and digested with recombinant thrombin (Novagen) for 3 h at room temperature. The tag and some remaining undigested protein were removed by incubation with nitrilotriacetic acid-agarose. The BPV1 E2 protein was expressed in yeast cells and purified as described previously (14, 36).

Nuclear extracts and in vitro transcription. HeLa S3 cell growth and preparation of nuclear extracts were performed as previously described (36). In vitro transcriptions were carried out in a total volume of 20 μl in 12 mM HEPES–12% glycerol–2 mM DTT–0.12 mM EDTA–40 mM KCl–7.5 mM $MgCl_2$–1 μl of RNasin (Promega). Supercoiled templates, the amounts of which are indicated in the figure legends, were preincubated with purified E2 protein, or only buffer when no protein was added, for 10 min at room temperature, 4 to 7 μl of nuclear extract was added, and incubation continued for 10 min to allow preinitiation complex formation. Transcription was initiated by the addition of nucleoside triphosphates (0.6 mM each). After 50 min, the reaction mixture was further incubated in the presence of 1 μl of DNase I for 10 min and stopped with 200 μl of stop solution (1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 140 mM NaCl, 4 μg of proteinase K, 4 μg of yeast tRNA) and incubated for 20 min at 37°C. After extraction by phenol-chloroform and precipitation, the RNA was resuspended in 10 μl of 225 mM KCl and the ^{32}P-phosphorylated primer was annealed by sequential incubation for 10 min at 65°C, 50 min at 55°C, and 10 min at room temperature. Primer extension was carried out for 60 min at 37°C in 50 mM Tris (pH 7.4)–75 mM KCl–3 mM $MgCl_2$–20 mM DTT–1 mM nucleoside triphosphates–0.08 mg of actinomycin D per ml–50 U of Superscript (Gibco BRL). The DNA was purified and analyzed on a 6% sequencing gel.

DNA binding assays. Double-stranded oligonucleotides were used as probes in the gel shifts to determine the affinity of E2. They encode the four E2 binding sites and some of the flanking sequences, with the following sequences (the E2 binding motif is underlined): BS-4, 5′ TGCTGTGCAACCGATTTCGGTTGC CTTTGGCTTATGT 3′; BS-3, 5′ AGTTTGCAACCGAAATAGGTTGGGCA GCAC 3′; BS-2, 5′ ATAAAAAAGGGAGTAACCGAAAACGGTCGGGACC GAA 3′; and BS-1, 5′ CGGTCGGGACCGAAAACGGTGTATATTAAAA GAT 3′. Probes, labeled by fill-in reactions using [^{32}P]ATP, were incubated with dilutions of the E2 proteins under reaction conditions as described before (36) except that 1 μg of poly(dI-dC) was used as the competitor instead of poly(G-C) and incubation was performed for 15 min on ice. The complexes were resolved on a 0.5× Tris-borate-EDTA–5% native polyacrylamide gel (29:1). The radioactive signals were quantified with the use of a PhosphorImager, and the free E2 concentration or the percentage of DNA bound by E2 was calculated. The apparent equilibrium constants given in Fig. 3A were determined as done previously for HPV16 E2 (32). The data were then used to generate a standard binding curve from which the free protein concentration, when 50% of the probe is shifted, could be determined. The free E2 concentration when 50% of BS-4 was bound was divided by the free protein concentration when 50% of the other probes were bound to determine the relative affinity. The relative affinities given in Fig. 3A represent the averages of four different experiments with independent labeling of the probes and two preparations of HPV18 E2 protein. For DNase I footprints, the binding reaction was carried out under the conditions described above in a total volume of 10 μl. DNase I digestion and analysis were performed as previously described (36).

RESULTS

The P105 promoter of HPV18 can be activated by E2 in vitro. To test whether the E2 protein of HPV18 can act as a transcriptional transactivator in in vitro transcription, as has been shown for E2 of BPV1 (14) and HPV16 (49), we prepared crude HeLa nuclear extracts and expressed in bacteria the E2 of HPV18 with six histidines at its amino terminus and partially purified the protein as described previously (36). An SDS-gel revealed a major 45-kDa band, which represents HPV18 E2, since it migrated slightly faster after removal of the tag by thrombin treatment (Fig. 2A). Furthermore, gel shift experiments confirmed that it was able to recognize a consensus E2 binding site (data not shown). The concentration of the binding-active protein was estimated to be 30 ng/μl (data not shown). As a template, we used a supercoiled CAT plasmid

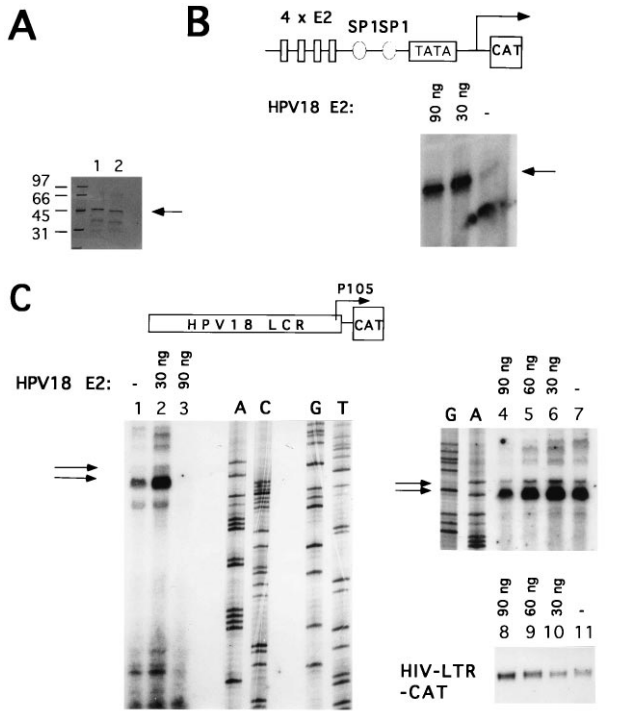

FIG. 2. In vitro transcription with HPV18 E2 and HeLa nuclear extracts. The RNA was analyzed by primer extension using a CAT primer. (A) An arrow indicates the position of the His-tagged HPV18 E2 protein (lane 1) and the E2 protein, where the tag was removed (lane 2), which were analyzed together with the molecular mass markers on an SDS-polyacrylamide gel and stained by Coomassie brilliant blue. Sizes are indicated in kilodaltons. (B) One hundred nanograms of the supercoiled template containing the synthetic promoter in front of the CAT gene, which is schematically drawn, was used in in vitro transcription either without E2 (−) or with the amounts indicated above the lanes. The arrow points to the transcripts initiating at the correct position. (C) Five hundred nanograms of supercoiled template, with the LCR of HPV18 in front of the CAT gene as shown, was used with no E2 (lane 1) or 30 ng (lane 2) or 90 ng (lane 3) of E2, or 750 ng was used with no E2 (lane 7) or 30 ng, 60 ng, and 90 ng (lanes 4 to 6) of purified HPV18 E2. A sequence of the template plasmid generated with the same primer was comigrated as a marker, with the A, C, G, and T lanes indicated. The arrows indicate the positions of the two transcripts initiating at the correct sites. As a control, 300 ng of an HIV LTR-CAT construct was assayed in the absence of E2 (lane 11) or in the presence of 30, 60, and 90 ng of E2 (lanes 10, 9, and 8).

with a synthetic promoter composed of four E2 binding sites, in addition to two binding sites for SP1 in front of the TATA box of the adenovirus major late promoter (p4E2Smlt) (Fig. 2B), which was strongly activated by BPV1 E2 (20) and by HPV18 E2 in transient cotransfection experiments (data not shown). The RNA 5′ end was mapped by primer extension with a primer hybridizing in the beginning of the CAT open reading frame (ORF). Figure 2B shows that the basal activity of this promoter was very low due to the low concentrations of the template DNA used in the experiment. Addition of 30 ng of purified E2 protein resulted in a 10-fold stimulation of the promoter, as quantified by a PhosphorImager. Increasing the amount of E2 protein to 90 ng did not further increase promoter activity; the promoter was stimulated eightfold. These experiments show that E2 of HPV18 is able to activate transcription in vitro.

In transfection experiments, inconsistent results have been obtained concerning the role of HPV18 E2 in the regulation of the HPV18 P105 promoter (5, 10, 47). We therefore investigated the effect on the P105 promoter mediated by binding of E2 to the LCR of HPV18 in in vitro transcription. The addition of purified protein to the reaction allows the exact control of the amount of E2, which may be more difficult in transient transfection assays. The template 4321-CAT (the numbers indicate the presence of the four E2 binding sites) contains the entire HPV18 LCR (positions 6929 to 120), with the CAT ORF under control of the P105 promoter. As shown in Fig. 2C, primer extension analysis of the RNA produced in vitro revealed two major initiation sites, at positions 105 and 103/102. These two species of transcripts deriving from the P105 promoter have also been identified in cells after transfection with this plasmid (31). When 30 ng of E2 was included in the transcription reaction, the amount of transcripts produced by the P105 promoter increased, as shown in Fig. 2C (lane 2). In a transcription reaction mixture containing 90 ng of E2, transcripts initiating at P105 could not be detected, suggesting that the promoter was completely repressed (Fig. 2C, lane 3). In another experiment, we used 750 ng of template, in contrast to 500 ng in the experiment described above, to reproduce this effect in smaller steps: the promoter was stimulated by adding 30 ng of E2. In the presence of 60 and 90 ng, the promoter started to be repressed (Fig. 2C, lanes 4 to 7). To confirm that the dose-dependent regulation by E2 is specific for the LCR of HPV18, transcription reactions were performed in parallel with the human immunodeficiency virus (HIV) long terminal repeat (LTR) in front of CAT, a template containing no E2 binding sites. Upon addition of 30 ng of E2, the amount which stimulated the P105 promoter, no effect on the activity of the LTR was observed. In the presence of 60 to 90 ng of E2, the LTR started to be activated moderately, in contrast to the repression of the HPV18 LCR (Fig. 2C, lanes 2 to 7 versus lanes 8 to 11). The stimulation of the LTR probably reflects some nonspecific activation in the presence of higher E2 concentrations. This nonspecific activation was also observed previously in transient transfection experiments and was shown to be independent of a DNA-binding function of E2 (21, 23).

These experiments show that at low concentrations, E2 stimulates P105 in vitro 2- to 4.5-fold, as determined in several independent experiments. Increasing the concentration of E2 reduces the promoter activity, with a complete repression in the presence of high amounts of E2.

Different affinities of E2 of HPV18 for its four sites present in the LCR. Activation of P105 at low concentrations and repression at high amounts, as described above, might be possible if E2 binding sites, which are involved in repression, are occupied only at higher E2 concentrations. Consequently, HPV18 E2 should bind with different affinities to its binding sites present in the LCR of HPV18. To analyze the relative equilibrium constants, we used oligonucleotides covering all four native E2 sites including 6- or 7-nucleotide flanking sequences as probes in gel shift assays with HPV18 E2. Figure 3A shows an example of a gel shift with 0.5 pmol of labeled oligonucleotides, encoding the four E2 binding sites as probes, incubated with 0.75 and 0.375 ng of E2. In the presence of the lower E2 concentration, 55% of the oligonucleotide encoding BS-4 was bound, whereas in the case of BS-1, only 16% was bound (Fig. 3A). These experiments suggested that E2 might have the highest affinity for BS-4. To quantify the relative affinities, we incubated several dilutions of E2 with fixed amounts of probe and analyzed the binding in gel shifts. After quantification of the radioactive signals, a standard binding curve was established to determine the free protein concentration when 50% of the probe is bound. It could be confirmed that E2 bound most strongly to BS-4. We used this value as a standard and calculated the binding strengths of the other binding sites relative to that of BS-4 (Fig. 3A). The advantage of this method is that the measurement is independent of the

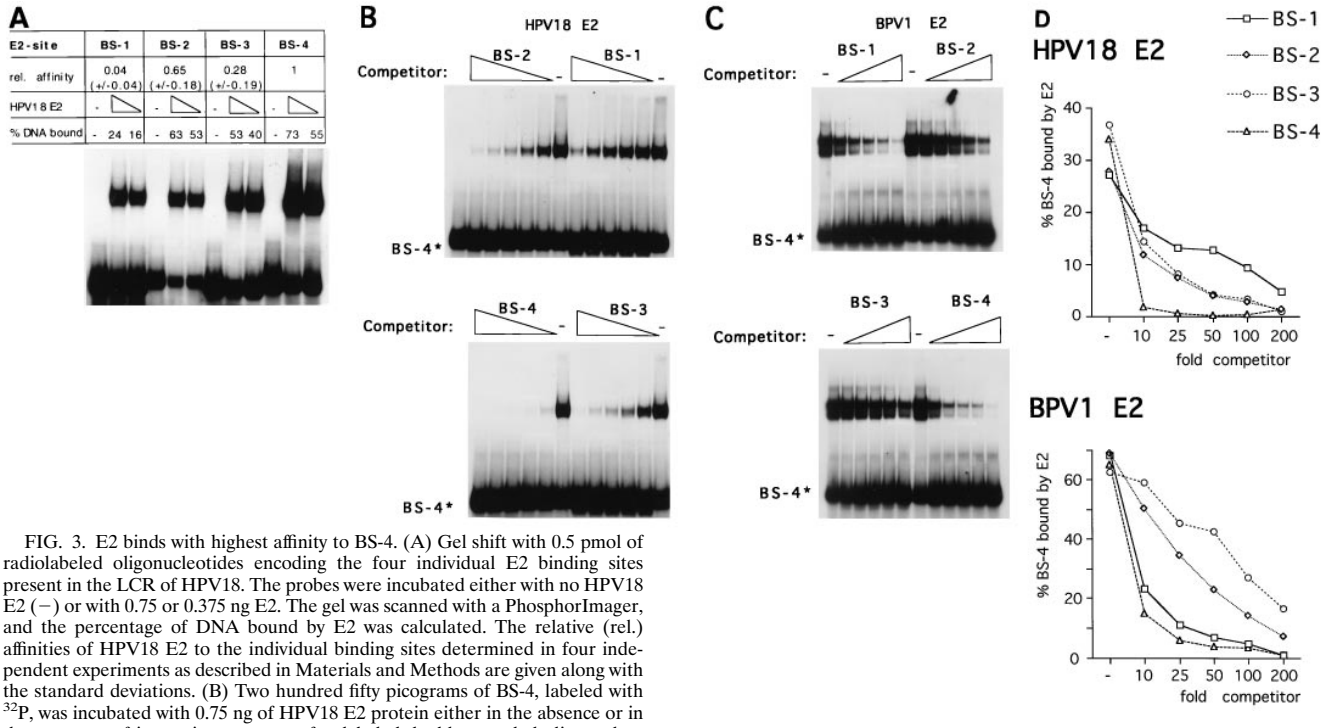

FIG. 3. E2 binds with highest affinity to BS-4. (A) Gel shift with 0.5 pmol of radiolabeled oligonucleotides encoding the four individual E2 binding sites present in the LCR of HPV18. The probes were incubated either with no HPV18 E2 (−) or with 0.75 or 0.375 ng E2. The gel was scanned with a PhosphorImager, and the percentage of DNA bound by E2 was calculated. The relative (rel.) affinities of HPV18 E2 to the individual binding sites determined in four independent experiments as described in Materials and Methods are given along with the standard deviations. (B) Two hundred fifty picograms of BS-4, labeled with ^{32}P, was incubated with 0.75 ng of HPV18 E2 protein either in the absence or in the presence of increasing amounts of unlabeled double-stranded oligonucleotides encoding E2 binding site BS-1, BS-2, BS-3, or BS-4 as indicated. (C) Two hundred fifty picograms of labeled BS-4 was incubated with 1 ng of purified BPV1 E2 protein and with the same amounts of unlabeled double-stranded oligonucleotides, encoding for the four E2 binding sites present in the HPV18 LCR, as in panel B. (D) The percentages of HPV18 E2 and BPV1 E2 bound to BS-4 were calculated and plotted against the amount of unlabeled oligonucleotide added as competitor.

concentration of active protein present in the preparation and of the specific activities of the labeled probes, which may vary from experiment to experiment and from probe to probe. In repeated assays, it was determined that the relative affinity to BS-2 was 0.65, that to BS-3 was 0.28, and that to BS-1 was 0.04 compared to the affinity to BS-4.

To confirm these values, we tested the four E2 binding sites for the ability to compete for binding of E2. In Fig. 3B, a fixed amount of E2 protein was incubated with labeled BS-4 in the presence of increasing amounts of unlabeled oligonucleotides encoding the four E2 binding sites. The plot of the percentage of BS-4 bound by E2 versus the amount of competitor DNA revealed that BS-4 was able to compete very well, whereas BS-1 was a much less efficient competitor (Fig. 3D) and BS-2 and BS-3 were intermediate. The E2 protein used in these studies contains six histidines fused at its N terminus to facilitate purification. To exclude that the presence of the tag might affect the E2 binding, we removed the tag from the purified protein by cleavage with thrombin, since a thrombin cleavage site between the six histidines and the ATG of HPV18 E2 is present. Repeating the gel shifts with the untagged protein gave the same results as shown in Fig. 3B and D, demonstrating that the E2 binding is not affected by the presence of the tag at its N-terminal part (data not shown).

The BPV1 E2 protein consistently was a strong repressor of HPV18 early gene expression, in contrast to the homologous protein (10, 14, 49). To test whether these functional differences might be related to DNA binding, we analyzed the binding strength of BPV1 E2 and compared it to that of the different E2 binding sites present in the LCR of HPV18. In gel shift reactions, a fixed amount of BPV1 E2 protein, expressed and purified from yeast cells as previously described (36), was incubated with radioactive labeled BS-4 in the presence of increasing amounts of unlabeled oligonucleotides encoding BS-1, BS-2, BS-3, and BS-4 (Fig. 3C). The corresponding plot, shown in Fig. 3D, illustrates that BPV1 E2 bound with highest affinity to BS-4, similar to HPV18 E2. Surprisingly, the BPV1 E2 protein bound much more strongly to BS-1 than to BS-2 and hardly at all to BS-3 (Fig. 3C and D).

To exclude that the affinity of the E2 proteins to the binding sites in HPV18 might be modulated by cooperative effects in the presence of several E2 sites, which would not have been observed in gel shifts using oligonucleotides with isolated E2 binding sites, we performed footprint experiments with the LCR of HPV18. A fragment encoding the entire LCR from positions 7003 to 120 (Fig. 1) was asymmetrically labeled and incubated with increasing amounts of HPV18 E2 and BPV1 E2. Figure 4A shows that both E2 proteins bound BS-4; the site was protected against DNase I digestion by all protein concentrations used. As indicated in Fig. 4A, BS-3 was bound very well by E2 of HPV18, whereas E2 of BPV1 did not bind at all to this sequence. To analyze in more detail the binding of E2 to BS-1 and BS-2, we used, in addition, another fragment encoding the LCR from positions 7572 to 120, thus encompassing E2 BS-1 to BS-3 (Fig. 1). Both proteins bound to the two promoter-proximal E2 sites simultaneously, although the footprints overlapped. Binding of E2 to BS-1 is best visible in the protection of the TA of the TATA box sequence (Fig. 4B). HPV18 E2 protected BS-1 only at the highest concentration, but BS-2 was occupied at the lowest concentrations. In contrast, even at the lowest concentrations, BS-1 was occupied by E2 of BPV1, which is again best visible in the protection of the TA of the TATA box. Furthermore, the lowest BPV1 E2 concentration is not sufficient to completely protect BS-2 (Fig. 4B). Thus, the footprint experiments confirm the results obtained by gel shifts.

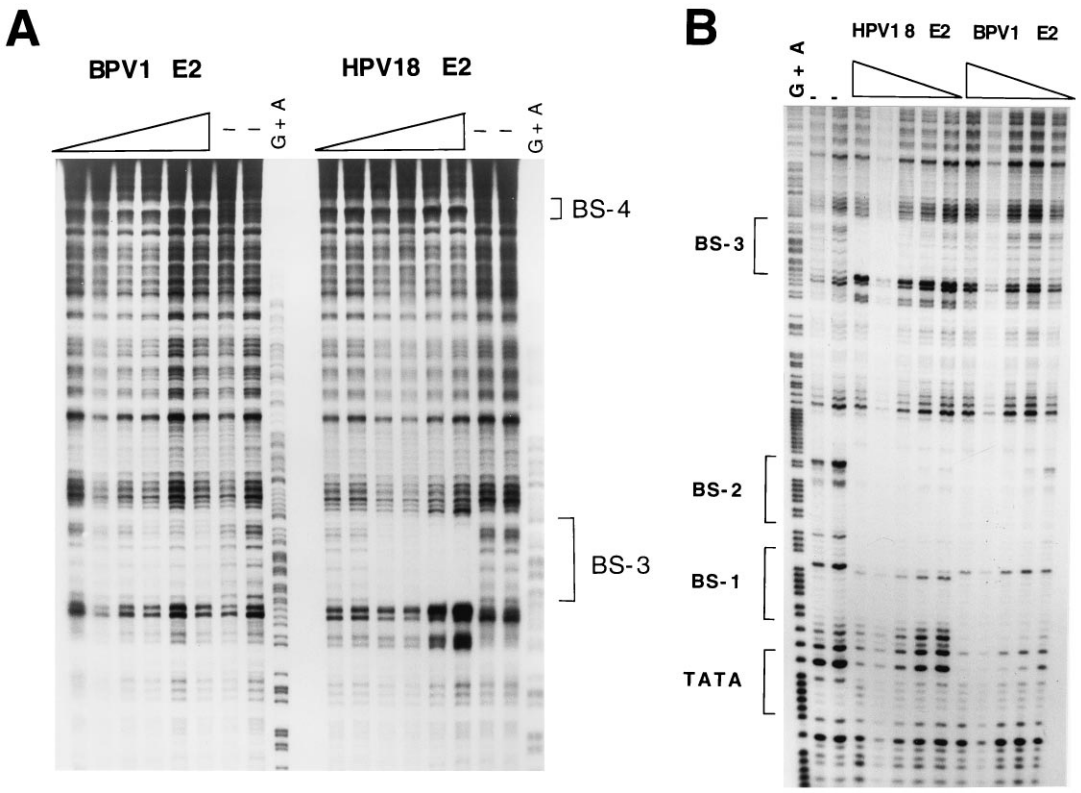

FIG. 4. Footprint analysis of E2 bound to the LCR of HPV18. Increasing amounts of HPV18 E2 and of BPV1 E2 were incubated with a labeled fragment encoding the LCR of HPV18, starting either at the *Sty*I site (position 7003) (A) or at the *Asc*I site (position 7572) (B), prior to digestion with DNase I. A G+A reaction of both fragments was used as a marker. The positions of the four E2 binding sites and the TATA box are indicated.

These in vitro binding studies revealed that both E2 proteins possess the highest affinities to BS-4. However, they differ in their affinities to the residual binding sites present in the HPV18 LCR. BS-3 represents a rather strong motif for HPV18 E2, whereas it is hardly recognized by BPV1 E2. In addition, the proteins differ in their affinities to BS-1, which is a strong motif for BPV1 and a much weaker for HPV18 E2.

Positive role of E2 binding site 4 in regulation of P105 activity in vivo. The DNA binding studies presented above indicate that depending on the intracellular E2 concentration, the occupation of the four E2 binding sites might vary. In the case of HPV18 E2, at low E2 concentrations BS-4 will be bound and increasing E2 concentrations will lead to occupancy of BS-2 and BS-3 and finally of BS-1. To verify whether E2 binding to BS-4 was responsible for the activation that we observed in transcription in vitro with low amounts of HPV18 E2, we analyzed the effect of BS-4 on the activity of P105 in transient transfection experiments. Two single-base substitutions in BS-4, which were shown to abolish binding of E2 (39, 47), were introduced into the wild-type HPV18 LCR. We cotransfected C33 cells, an HPV-negative cervical carcinoma cell line, with increasing amounts of an expression vector for HPV18 E2 and 2 μg of a reporter plasmid containing either the entire HPV18 LCR in front of the luciferase gene, called 4321-Luc, or an LCR with the mutation in BS-4, X321-Luc. Increasing amounts of HPV18 E2 reduced the promoter under control of the wild-type LCR. For example, in the presence of 100 ng of E2 expression vector, 62% of the initial activity of the promoter remained (Fig. 5A). The mutation of BS-4 in X321-Luc more strongly reduced the luciferase activity when the amount of E2 was increased. One hundred nanograms of E2 expression vector left only 30% of the basal activity. Upon transfection of 250 ng of E2 expression vector, P105 under control of the HPV18 LCR with mutations in all four E2 binding sites (XXXX-Luc) also was repressed, suggesting that in this case, nonspecific repression probably reflecting squelching due to nonphysiological amounts of E2 (17), might be involved.

Previous reports suggested that E2 binding to BS-1 might be sufficient for repression of the early promoter in the case of HPV18 (10). Since our binding studies revealed that BPV1 E2 and HPV18 E2 differ in their relative affinities for BS-1 (Fig. 3 and 4), we compared the effects of BPV1 E2 over a large spectrum of concentrations with that of HPV18 E2 on P105 activity. As shown in Fig. 5B, transfection of very little BPV1 E2 expression vector (pC59) resulted in a threefold stimulation of P105 activity in the wild-type LCR, whereas a further increase of the E2 concentration induced promoter repression. In the presence of 400 ng, a 70% reduction of the promoter function was observed. Activation in the presence of low BPV1 E2 levels was lost when the LCR had a mutated BS-4 (X321-Luc). As seen in Fig. 5B, the promoter in this construct was repressed by transfecting 10 ng of expression vector. The role of BS-4 was most obvious with 200 ng of expression vector: the wild-type promoter 4321 was only marginally repressed, whereas X321 retained only 15% of the basal activity (Fig. 5B).

The transfection experiments summarized in Fig. 5A and B indicate that BPV E2 is able to repress P105 more strongly than HPV18 E2, which correlates with our finding that BPV1 E2 binds more strongly to BS-1 than HPV18 E2. Furthermore, binding of E2 to BS-4 has a stimulatory effect on the activity of P105 in vivo. Activation by low amounts of BPV1 E2 is due to

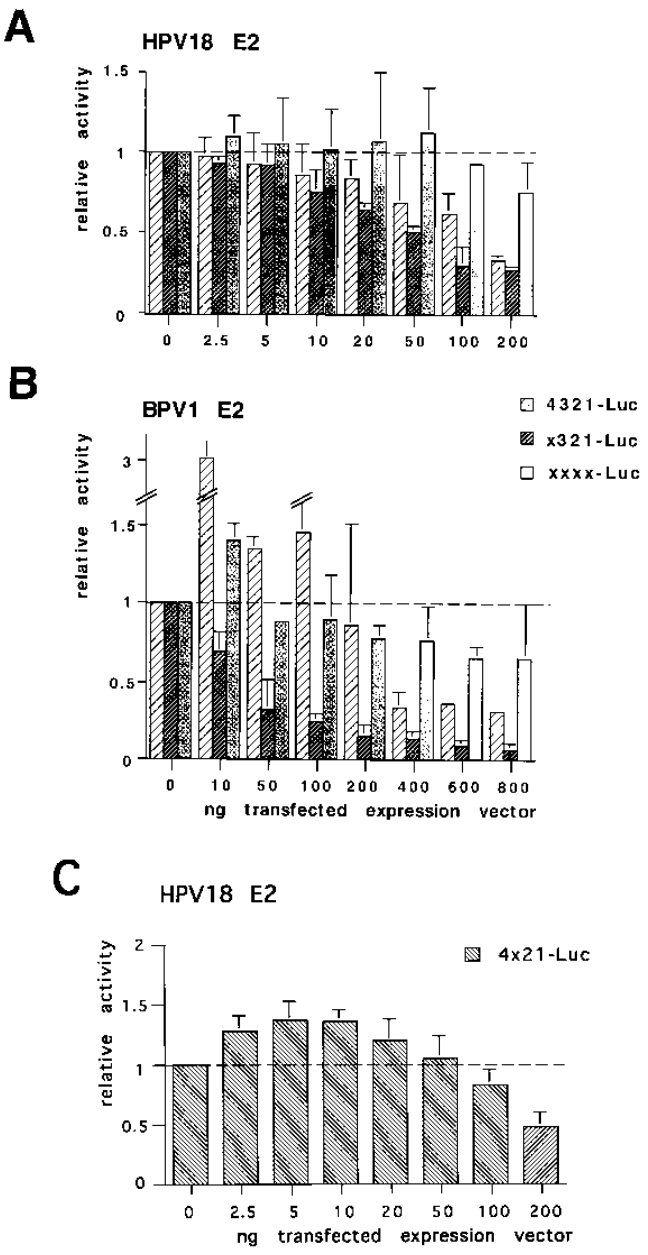

FIG. 5. Binding of E2 to BS-4 has a positive effect on the activity of the P105 promoter in vivo. C33 cells were cotransfected with 2 μg of reporter plasmids and increasing amounts of expression vectors for HPV18 E2 under control of the CMV immediate-early promoter (A and C) or BPV1 E2 under control of the SV40 early promoter (B). The reporter plasmids contain the wild-type LCR in front of the luciferase gene, 4321-Luc (the number corresponds to the presence of all four E2 binding sites), the HPV18 LCR with a point mutation in BS-4, X321-Luc, the LCR with mutations in all four E2 binding sites, XXXX-Luc, or the LCR with mutation in BS-3, 4X21-Luc (C). The luciferase activities were normalized by β-galactosidase activities; the fold stimulation by E2 was calculated and given as relative activities. These values, which were plotted against the amount of expression vector cotransfected, represent the means of at least five individual experiments. Standard deviations are indicated. In cases without error bars, the standard deviation is 0.

binding to BS-4, and as shown for both E2 proteins, even in the presence of high E2 concentrations, where all E2 binding sites should be occupied, E2 bound on BS-4 counteracts the repression conferred by E2 occupying residual sites.

However, in contrast to transcription in vitro, we have not been able to detect activation by HPV18 E2 in vivo with the wild-type LCR of HPV18 under conditions similar to those where we observed activation by the BPV1 E2 protein. There are two differences between the transfections with HPV18 E2 and BPV1 E2 which should be taken into account. As revealed by gel shifts and footprint analysis, HPV18 E2 bound very well to BS-3, whereas the BPV1 protein failed to recognize this sequence. Furthermore, both expression vectors contain heterologous promoters with different strengths, which could give rise to different levels of proteins present in the transfected cells.

The role of BS-3 in transcriptional regulation of P105 is not very well characterized, although previous experiments suggested it might have a repressing effect (47). To investigate whether the activation observed with small amounts of BPV1 E2 might be related to the lack of binding to BS-3, we introduced a point mutation within the context of the wild-type LCR to abolish HPV18 E2 binding to BS-3. The result of cotransfections with increasing amounts of HPV18 E2 expression vector and 2 μg of this reporter construct, 4X21-Luc, showed that P105 was slightly activated by small amounts of HPV18 E2, followed by a dose-dependent repression (Fig. 5C). This points to a negative effect mediated by E2 bound on BS-3.

As already mentioned, the expression vectors for E2 of BPV1 and HPV18 contain different promoters, the strong cytomegalovirus (CMV) immediate-early promoter/enhancer (4) directing expression of HPV18 E2 and the weaker SV40 early promoter in the case of BPV1 E2. Although the number of cells transfected with the HPV18 E2 expression vector might be reduced by using small amounts of DNA, the single cells that have been transfected might produce enough HPV18 E2 protein due to the strong CMV promoter. Thus, the intracellular E2 concentration might always be higher as required for saturation of BS-4 only. In other words, in vivo we would have already observed repressed transcription. In contrast, the weaker SV40 early promoter, which directs the expression of the BPV1 E2 protein, might allow the production of subsaturating amounts of E2 in the transfected cells. To test this hypothesis, we increased the amount of reporter plasmid from 2 to 3 μg and cotransfected a similar spectrum of HPV18 E2 expression vector as above. Under these conditions, the ratio of E2 protein to HPV18 LCR should be reduced in the transfected cells. As shown in Fig. 6, small amounts of E2 expression vector resulted in a moderate but significant stimulation of the E6/E7 promoter, which was on average 1.6-fold. This activation was due to the presence of BS-4, since the construct X321-Luc was not activated significantly. To further demonstrate that binding of E2 to BS-4 was sufficient to activate P105, we tested the construct 4XXX-Luc, with a single binding site, BS-4, intact. In the presence of 15 ng of E2, the activity of the promoter was stimulated 2.1-fold and remained at this level when the E2 concentration was increased, in contrast to the wild-type construct.

DISCUSSION

The E2 proteins of PV are transcriptional transactivator proteins with very high affinities for their binding sites; the values determined for E2 of BPV1 and HPV16 vary from 10^{-9} to 8×10^{-11} (32, 42, 49). Thus, already present in low concentrations in the cells infected with HPVs or transfected with an E2 expression vector, E2 might occupy its sites present in the LCR of the virus or in a reporter construct. To analyze the role of E2 in regulation of the E6/E7 promoter of HPV18 in the presence of subsaturating amounts, we set up an in vitro transcription system which allowed the study of a dose re-

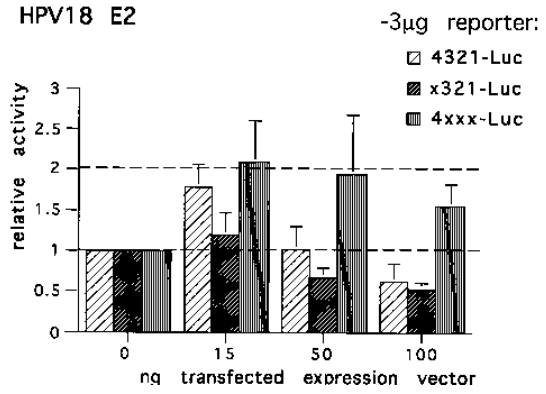

FIG. 6. Three micrograms of reporter plasmid 4321-Luc, X321-Luc, or 4XXX-Luc, which contains the HPV18 LCR with BS-4 intact, were used in cotransfection experiments. The relative activities were determined and plotted against the amount of HPV18 E2 expression vector which was cotransfected. The values represent the means of five independent experiments, with all points in duplicate. The standard deviations are given. The difference in activity between 4321-Luc and X321-Luc in the presence of 15 ng of E2 expression vector is significant.

sponse to E2 on the LCR of HPV18. As a source for general transcription factors, we used nuclear extracts made from HeLa cells, representing natural target cells for genital HPVs. HeLa cells contain HPV18 DNA integrated into the cellular genome; however, the E2 ORF has been disrupted by integration; thus, endogenous E2 will not interfere with our experiments (35). In vitro, HPV18 E2 expressed and purified from bacteria could strongly activate a synthetic promoter, containing two binding sites for SP1 in addition to four E2 binding sites, suggesting that the purified protein is a strong transcriptional transactivator per se, acting directly on the assembly of the preinitiation complex.

In vitro transcription revealed that E2 activates P105 at low concentrations, whereas increasing the concentration results in complete promoter repression. The repression that we observed in vitro was not due to squelching, as the heterologous promoter containing E2 sites in activator positions was still stimulated by the same amount of E2 and the activity of an unrelated promoter without E2 binding sites, the HIV LTR, was not repressed by these E2 concentrations in vitro (Fig. 2B and C, lanes 8 to 11). A series of experiments showed that both promoter proximal E2 binding sites are involved in repression of the early promoters of HPV11, HPV16, and HPV18, where binding of E2 interferes with the binding of TBP and SP1 (10, 12, 31, 42, 47). The repression that we observed probably involves the same mechanism. The specificity of the repression due to E2 binding to its sites was also demonstrated in transient cotransfection assays. We used, in parallel to the wild-type LCR, a reporter construct with an LCR which had mutations of all four E2 binding sites (Fig. 5A and B) to find conditions where only the wild-type promoter was repressed by cotransfecting increasing amounts of E2. Therefore, we could be sure that repression was due to binding of E2 to its binding sites and not to nonspecific repression by squelching of transcription in the presence of nonphysiological high amounts of E2 (17). We found 70% specific repression of the P105 induced by BPV1 E2 and 40% induced by HPV18 E2 in the presence of the wild-type LCR. In agreement with our results, almost complete repression of the LCR of HPV18 was obtained by transfecting BPV1 E2, but repression was weaker with the homologous E2 protein (3, 10, 44, 47). Inconsistent results concerning the modes of action of homologous E2 proteins and BPV1 E2 have also been described for other PV types (5, 25). The enhanced repression by BPV1 E2 correlates with our data obtained in binding studies, which revealed that the BPV1 E2 protein had a higher relative affinity to BS-1 compared to that of the HPV18 E2. The increased binding to BS-1, which has been shown to be sufficient for promoter repression in the case of HPV18 (10), might enable the BPV1 E2 protein to be more efficient in displacing TFIID (14). However, we certainly cannot exclude the possibility that the distinct mode of action that we observe between E2 of HPV18 and BPV1 is due to additional functional differences present in other domains of the proteins.

We succeeded in finding conditions under which both E2 proteins stimulated P105 at low concentration in vivo and could thus confirm the data obtained by transcription in vitro. The limit to control the E2 concentration in in vivo transfection assays became obvious, since we were not able to decrease the protein produced in the cells to such a level that mostly BS-4 was occupied. Only when we increased the number of E2 binding sites by raising the concentrations of the reporter construct did activation by HPV18 E2 become obvious. The activation of P105 observed at low concentrations was due to binding of E2 to the most distal E2 BS-4, since mutation of BS-4 led to loss of activation. The stimulating role of BS-4 was also evident in the presence of elevated E2 concentrations; binding of HPV18 E2 and BPV1 E2 to BS-4 counteracted repression (Fig. 5A and B). A recent study suggested that binding of E2 to BS-2 might play a positive role in the activity of P105 (10). In our experiments, BS-4 was the sole binding site necessary for activation, since the construct with mutations in BS-4 was no longer activated significantly. We could also demonstrate that a single binding site, BS-4, was sufficient to activate P105. Transfections with the 4XXX-Luc reporter construct led to twofold stimulation (Fig. 6). In studies of the mechanism of activation by the BPV1 E2 protein, it has been shown that the presence of one E2 palindrome activates transcription only weakly in mammalian cells, while two sites can function efficiently as an E2-dependent enhancer (16, 22, 45). Activation in the presence of one E2 site was about two- to sixfold (16, 45), which is in the same range as we observed here in vitro and in vivo. The stronger activation observed by BPV1 E2 compared to HPV18 E2 might partially be due to the fact that BPV1 E2 does not bind to BS-3, which is also involved in promoter repression (Fig. 5C). However, there may be functional differences between BPV1 E2 and HPV18 E2 in the transactivation domain that might also account for the difference.

In vitro binding studies revealed that E2 has the highest affinity to BS-4 and reduced affinity to repressive sites, which correlates perfectly with the dose-dependent regulation of P105 activity by E2. Nevertheless, the results obtained in binding studies are somewhat surprising. First, although BS-1 and BS-2 contain the perfect palindromic sequence ACCGAAAA CGGT and the same A/T-rich internal sequence, which was suggested to increase the binding (39), the affinity of E2 differed by a factor of 25 (Fig. 3A). Apparently, the composition of the flanking sequences may strongly influence the affinity, as was observed to a lower extent in the case of BPV1 E2 (26). Second, E2 of HPV18 and BPV1 showed very different patterns of binding to BS-3, which has a degenerated palindromic sequence, ACCGN_4AGGT. BS-3 was hardly bound by BPV1 E2, but it represented a strong motif for HPV18 E2. In the case of BPV1 E2, it is known that the perfect palindromic sequence is important for binding with high affinity (26). However, it seems that the HPV18 E2 might have amino acid changes in its DNA binding domain, which tolerate a substitution in the

palindromic recognition sequence. The distinct DNA-binding properties of the two E2 proteins were also obvious at BS-1. Gel shifts and footprints shown in Fig. 3 and 4 clearly demonstrated that BPV1 E2 has a higher affinity for BS-1 than HPV18 E2. Comparing the affinities of HPV16 E2 and BPV1 E2 to the equivalent HPV16 E2 binding site, the HPV16 protein also bound more weakly than the BPV1 E2 protein (49). Thus, it might be possible that the E2 proteins of a particular PV type have adopted specific DNA-binding properties, although the proteins are highly conserved in their DNA binding domains and they recognize the same consensus sequence.

Dose-dependent regulation of PV gene expression due to variable occupancy of E2 binding sites has been described for regulation of the HPV8 late promoter and BPV4 early gene expression. In these cases, repression of transcription by the full-length E2 protein occurs only at high concentrations, when lower-affinity sites which overlap with binding sites for cellular factors are also bound. At lower E2 concentrations, only the activating sites are occupied (25, 39, 40). We describe here that differential regulation of early gene expression by E2 might also occur in genital HPVs, although the differences in affinity of the binding sites seemed to be much less than in the case of HPV8 and BPV4. Studying the binding affinity of HPV16 E2 to its binding sites in the LCR of HPV16, Sanders and Maitland (32) found only minor differences in the apparent equilibrium constants. They used an E2 protein lacking the N-terminal domain, which might modulate the DNA binding activity, in contrast to our study, in which the full-length E2 protein active in transcription was used. Interestingly, the half-lives of the DNA-E2 complexes showed great differences in HPV16. The binding site equivalent to BS-4 was bound most stably (32), suggesting that at low E2 concentrations, this distal E2 binding site might be occupied primarily, similar to the situation that we suggest here for HPV18.

Our data suggest a new role for E2 in the regulation of early HPV18 gene expression, which involves both activation and repression. HPV early gene expression will be initially stimulated by the epithelial cell-specific enhancer (reviewed in reference 48). Then, E6 and E7 proteins push the cell into the cell cycle to allow synthesis of cellular components required for DNA replication (6). Low amounts of E2 will further activate the promoter by binding to BS-4 and generate more early proteins, including E2, which will start to occupy residual E2 sites. Binding of E2 to BS-1, -2, and -3 leads to repression of transcription of the early promoter and, in parallel, to activation of viral DNA replication, since these E2 binding sites, which are involved in transcriptional silencing, are required for full viral DNA replication (9, 41). However, as suggested by our data, transcription will not be switched off but will be maintained on a certain level due to binding of E2 to BS-4, which counteracts strong repression. Transcription of the early promoter might even be reactivated after a decrease in the E2 concentration, and alternation between activation of transcription and of replication occurs. In this way, the amount of the viral factors E1 and E2, which are required for replication, would increase in parallel to the number of replicating viral genomes during the lytic phase of viral infection. Furthermore, the expression of E6 and E7 would also be kept at a level necessary for maintaining a replication-competent cellular environment.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1992. Short protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York, N.Y.
2. **Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61:** 962–971.
3. **Bernard, B. A., C. Bailly, M. C. Lenior, M. Darmon, F. Thierry, and M. Yaniv.** 1989. The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J. Virol. **63:**4317–4324.
4. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41:**521–530.
5. **Bouvard, V., A. Storey, D. Pim, and L. Banks.** 1994. Characterization of the human papillomavirus E2 protein: evidence of *trans*-activation and *trans*-repression in cervical keratinocytes. EMBO J. **13:**5451–5459.
6. **Cheng, S., D.-C. Schmidt-Grimminger, T. Murant, T. Broker, and T. L. Chow.** 1995. Differentiation dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. Genes Dev. **9:**2335–2349.
7. **Chin, M. T., T. R. Broker, and L. T. Cow.** 1989. Identification of a novel constitutive enhancer element and an association binding protein: implications for human papillomavirus type 11 enhancer regulation. J. Virol. **63:** 2967–2976.
8. **Chow, L., and T. Broker.** 1994. Papillomavirus DNA replication. Intervirology **37:**150–158.
9. **Demeret, C., M. Le Moal, M. Yaniv, and F. Thierry.** 1995. Control of HPV 18 DNA replication by cellular and viral transcription factors. Nucleic Acids Res. **23:**4777–4784.
10. **Demeret, C., M. Yaniv, and F. Thierry.** 1994. The E2 transcriptional repressor can compensate for SP1 activation of the human papillomavirus type 18 early promoter. J. Virol. **68:**7075–7082.
11. **DiMaio, D.** 1991. Transforming activity of bovine and human papillomaviruses in cultured cells. Adv. Cancer Res. **56:**133–159.
12. **Dong, G., T. R. Broker, and L. T. Chow.** 1994. Human papillomavirus type 11 E2 proteins repress the homologous E6 promoter by interfering with the binding of host transcription factors to adjacent elements. J. Virol. **68:**1115–1127.
13. **Dong, X.-P., F. Stubenrauch, E. Beyer-Finkler, and H. Pfister.** 1994. Prevalence of deletions of YY1-binding sites in episomal HPV 16 DNA from cervical cancers. Int. J. Cancer **58:**803–808.
14. **Dostatni, N., P. F. Lambert, R. Sousa, J. Ham, P. M. Howley, and M. Yaniv.** 1991. The functional BPV-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex. Genes Dev. **5:**1657–1671.
15. **Dostatni, N., F. Thierry, and M. Yaniv.** 1988. A dimer of BPV-1 E2 containing a protease resistant core interacts with its DNA target. EMBO J. **7:**3807–3816.
16. **Gauthier, J. M., N. Dostatni, M. Lusky, and M. Yaniv.** 1991. Two DNA-bound E2 dimers are required for strong transcriptional activation and for cooperation with cellular factors in most cells. New Biol. **3:**498–509.
17. **Gill, G., and M. Ptashne.** 1988. Negative effect of the transcriptional activator GAL 4. Nature **334:**721–724.
18. **Giri, I., and M. Yaniv.** 1988. Structural and mutational analysis of E2 trans-activating proteins of papillomaviruses reveal three distinct functional domains. EMBO J. **7:**2823–2829.
19. **Ham, J., N. Dostatni, J. M. Gauthier, and M. Yaniv.** 1991. The papillomavirus E2 protein: a factor with many talents. Trends Biochem. Sci. **16:**440–444.
20. **Ham, J., G. Steger, and M. Yaniv.** 1994. Cooperativity in vivo between the E2 transactivator and the TATA box binding protein depends on core promoter structure. EMBO J. **13:**147–157.
21. **Haugen, T. H., L. Turek, F. M. Mercurio, T. P. Cripe, B. J. Olson, R. D. Anderson, D. Seidl, M. Karin, and J. Schiller.** 1988. Sequence-specific and general transcriptional activation by the bovine papillomavirus-1 E2 transactivator require an N-terminal amphiphatic helix-containing E2 domain. EMBO J. **7:**4245–4253.
22. **Hawley-Nelson, P., E. J. Androphy, D. R. Lowy, and J. T. Schiller.** 1988. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2 dependent enhancer. EMBO J. **7:**525–531.
23. **Heike, T., S. Miyatake, M. Yoshida, K.-I. Arai, and A. Naoka.** 1989. Bovine papilloma virus encoded E2 protein activates lymphokine genes through DNA elements distinct from the consensus motif, in the long control region of its own genome. EMBO J. **8:**1411–1417.
24. **Hoffmann, A., and R. G. Roeder.** 1991. Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acids Res. **19:**6337–6338.

25. **Jackson, M. E., and S. M. Campo.** 1995. Both viral E2 protein and the cellular factor PEBP2 regulate transcription via E2 consensus sites within bovine papillomavirus type 4 long control region. J. Virol. **69:**6038–6046.
26. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3:**510–526.
27. **McBride, A. A., J. C. Byrne, and P. M. Howley.** 1989. E2 polypeptides encoded by the bovine papillomavirus type 1 form dimers through the common carboxyl-terminal domain: transactivation is mediated by the conserved amino-terminal domain. Proc. Natl. Acad. Sci. USA **86:**510–514.
28. **McBride, A. A., H. Romanczuk, and P. M. Howley.** 1991. The papillomavirus E2 regulatory proteins. J. Biol. Chem. **266:**18411–18414.
29. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. McPherson, and M. R. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250:**1694–1699.
30. **O'Connor, M., S.-Y. Chan, and H.-U. Bernard.** 1995. Transcription factor binding sites in the long control region of genital HPVs, p. 21–40. *In* G. Myers, H. Delius, J. Icenogel, H.-U. Bernard, C. Baker, A. Halpern, and C. Wheeler (ed.), Human papillomaviruses 1995. Theoretical Biology and Biophysics, Los Alamos, N.Mex.
31. **Romanczuk, H., F. Thierry, and P. M. Howley.** 1990. Mutational analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P97 and type 18 P105 promoters. J. Virol. **64:**2849–2859.
32. **Sanders, C. M., and N. J. Maitland.** 1994. Kinetic and equilibrium binding studies of the human papillomavirus type-16 transcription regulation protein E2 interacting with core enhancer elements. Nucleic Acids Res. **22:**4890–4897.
33. **Schneider, A.** 1994. Natural history of genital papillomaviruses infections. Intervirology **37:**201–214.
34. **Schneider-Gädicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5:**2285–2292.
35. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, A. Roggenbuch, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature **314:**111–114.
36. **Steger, G., J. Ham, O. Lefebvre, and M. Yaniv.** 1995. The bovine papillomavirus 1 E2 protein contains two activation domains: one that interacts with TBP and another that functions after TBP binding. EMBO J. **14:**329–340.
37. **Steger, G., J. Ham, F. Thierry, and M. Yaniv.** 1993. The E2 proteins of papillomaviruses as transcription and replication factors, p. 263–278. *In* W. Doerfler and P. Boehm (ed.), Virus strategies. VCH, Weinheim, Germany.
38. **Stenlund, A., and M. R. Botchan.** 1990. The E2 trans-activator can act as a repressor by interfering with a cellular transcription factor. Genes Dev. **4:**123–136.
38a. **Stinski, M.** Personal communication.
39. **Stubenrauch, F., and H. Pfister.** 1994. Low-affinity E2 binding site mediates downmodulation of E2 transactivation of the human papillomavirus type 8 late promoter. J. Virol. **68:**6959–6966.
40. **Stubenrauch, F. L., and H. Pfister.** 1996. E2 represses the late gene promoter of human papillomavirus type 8 at high concentrations by interfering with cellular factors. J. Virol. **70:**119–126.
41. **Sverdrup, F., and S. A. Khan.** 1995. Two E2 binding sites alone are sufficient to function as the minimal origin of replication of human papillomavirus type 18. J. Virol. **69:**1319–1323.
42. **Tan, S.-H. L., E.-C. Louis, P. A. Walker, and H.-U. Bernard.** 1994. The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E2 promoter through displacement of SP1 and TFIID. J. Virol. **68:**6411–6420.
43. **Tan, S. H., B. Gloss, and H.-U. Bernard.** 1992. During negative regulation of the human papillomavirus-16 E6 promoter, the viral E2 protein can displace Sp1 from a proximal promoter element. Nucleic Acids Res. **20:**251–256.
44. **Thierry, F., and M. Yaniv.** 1987. The BPV1 E2 trans-acting protein can be either a repressor or activator of the HPV18 regulatory region. EMBO J. **6:**3391–3397.
45. **Thierry, F., N. Dostatni, F. Arnos, and M. Yaniv.** 1990. Cooperative activation by papillomavirus type 1 E2 can occur over a large distance. Mol. Cell. Biol. **10:**4431–4437.
46. **Thierry, F., M. J. Heard, K. Dartmann, and M. Yaniv.** 1987. Characterization of a transcriptional promoter of human papillomavirus 18 and modulation of its expression by simian virus 40 and adenovirus early antigens. J. Virol. **61:**134–142.
47. **Thierry, F., and P. Howley.** 1991. Functional analysis of E2-mediated repression of the HPV18 P105 promoter. New Biol. **3:**90–100.
48. **Turek, L. P.** 1994. The structure, function, and regulation of papillomaviral genes in infection and cervical cancer. Adv. Virus Res. **44:**305–356.
49. **Ushikai, M., M. J. Lace, Y. Yamakawa, M. Kono, J. Anson, et al.** 1994. Transactivation by the full-length E2 protein of human papillomavirus type 16 and bovine papillomavirus type 1 in vitro and in vivo: cooperation with activation domains of cellular transcription factors. J. Virol. **68:**6655–6666.
50. **Ustav, E., M. Ustav, P. Symanski, and A. Stenlund.** 1993. The bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional transactivator. Proc. Natl. Acad. Sci. USA **90:**898–902.
51. **Vousden, K. H.** 1994. Interactions between papillomavirus proteins and tumor suppressor gene products. Adv. Cancer Res. **64:**1–24.
52. **Werness, B. A., K. Münger, and P. Howley.** 1991. Role of the human papillomavirus oncoproteins in transformation and carcinogenic progression. Important Adv. Oncol. **1991:**3–18.
53. **Yang, L., R. Li, I. J. Mohr, R. Clark, and M. R. Botchan.** 1991. The bovine papillomavirus type 1 (BPV-1) replication protein E1 modulates transcriptional activation by interacting with BPV-1 E2. Nature **353:**628–632.
54. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82:**1030–1034.
55. **zur Hausen, H.** 1991. Viruses in human cancers. Science **254:**1167–1173.