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Mutational Analysis of *cis* Elements Involved in E2 Modulation of Human Papillomavirus Type 16 P_{97} and Type 18 P_{105} Promoters

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***cis*-Acting elements involved in E2 modulation of human papillomavirus type 16 (HPV-16) P_{97} promoter activity and HPV-18 P_{105} promoter activity were examined. In transfected primary human keratinocytes, each promoter had a basal activity that could be repressed by the bovine papillomavirus type 1 E2 gene product. Mutational analysis of the E2-binding sites in the long control region upstream of each promoter revealed that E2 repression was mediated through the E2-binding sites proximal to each promoter. In the context of a mutated E2-binding site at the promoter proximal position, the HPV-16 P_{97} and HPV-18 P_{105} promoters could be transactivated by E2. E2-mediated repression of HPV-18 P_{105} promoter activity was shown to be a transcriptional effect. The interaction of E2 with promoter-proximal E2-binding sites is likely to be important for the controlled expression of viral genes transcribed from the HPV-16 P_{97} promoter and the HPV-18 P_{105} promoter in infected human genital epithelial cells.**

A subgroup of the human papillomaviruses, which includes type 16 (HPV-16) and type 18 (HPV-18), has been found in the majority of anogenital carcinomas, including cervical carcinomas (for a review, see reference 56), suggesting that these viruses play an important role in the etiology of the carcinogenic progression of HPV-associated premalignant lesions of the genital tract. This hypothesis is further supported by the in vitro transforming potential of these same HPV types in studies with either cloned full-length viral DNA or specific subgenomic fragments of the viral genome expressed from heterologous promoters. HPV-16 DNA and HPV-18 DNA can immortalize primary human keratinocytes, inducing histological abnormalities characterized by altered patterns of cellular differentiation (11, 22, 30, 37, 39). The most detailed genetic and functional studies of HPV transformation have been done with HPV-16, but analogous studies with HPV-18 suggest that these two viruses may be similar with respect to viral gene functions.

Mutational analyses of HPV-16 have indicated that a subset of early viral genes have immortalization and transformation functions similar to those of the full-length genome. One of these genes, E7, encodes a protein that functionally and structurally resembles the adenovirus E1A gene product in that it can transcriptionally activate the adenovirus E2 promoter (36), can cooperate with *ras* to transform primary baby rat kidney cells (36, 50), and can form a complex with the retinoblastoma gene product (13, 34). The E6 and E7 open reading frame (ORF) gene products are required to immortalize primary human keratinocytes in culture (21, 33) and to induce histological changes similar to those imparted by the full genome (9). The histological changes and altered patterns of differentiation induced in vitro closely resemble those seen in genital neoplasia in vivo (30), further supporting an active role for these HPVs in the development of human neoplasia. Analyses of isolated HPV-18 ORFs suggest that the HPV-18 E6 and E7 gene products have similar transforming functions (4).

The E6 and E7 gene products have been detected in cells transformed by HPV-16 DNA in vitro (1) and in cell lines established from cervical carcinomas (44, 47). It appears that the mRNAs transcribed from these ORFs are either preferentially selected or retained in these cells, whereas transcripts encoded by other viral ORFs are often incomplete or undetected (3, 42, 47). Analyses of HPV-16-associated cervical lesions have shown differences in viral transcription patterns between precancerous lesions and invasive carcinomas (45). One factor important for viral transcription in cervical lesions appears to be the integrity of the viral genome. In benign lesions, HPV-16 DNA is extrachromosomal, whereas in cervical carcinomas, the DNA is usually integrated into the host genome (12, 41, 45). Analyses of HPV-18-associated lesions suggest that this viral genome is also often integrated in the carcinomas (40). The importance of the extrachromosomal state of the viral genomes in preneoplastic lesions compared with an integrated state in the carcinomas as a prerequisite, codeterminant, or outcome of transformation by these viruses is not known. Integration does not appear to be a random event with respect to the region of the viral genome which is disrupted. In tumors, as well as in cervical carcinoma cell lines, integration often interrupts E1 and E2 ORFs (3, 12, 42). The disruption of the E2 gene can be predicted to result in altered patterns of viral gene expression due to the subsequent absence of the E2 gene products involved in transcriptional regulation.

The full-length products of the E2 ORF of HPV-16 can conditionally modulate transcription (10, 35). On the basis of more thorough studies using bovine papillomavirus type 1 (BPV-1) E2, this regulation is thought to occur by the binding of full-length transactivator or smaller repressor proteins encoded by E2 to $ACCN_6GGT$ motifs within the viral genome (2, 8, 26, 31, 35). $ACCN_6GGT$ motifs are located within the HPV-16 long control region (LCR) upstream of the P_{97} promoter which directs the transcription of the E6 and E7 genes (47). The interaction of the E2 gene product(s) in this region may be involved in regulating the expression of the E6 and E7 transforming genes, and a disruption of the E2 ORF could therefore result in the loss of viral regulation of these genes. It has not yet been deter-

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mined whether the E2 ORF of HPV-16 encodes multiple regulatory proteins analogous to those of BPV-1, but modulation of promoter activity has been achieved by using cloned HPV-16 DNA corresponding to full-length and carboxy-terminal E2 proteins (10, 35). These results are consistent with a model of transactivation by full-length E2 proteins and repression by smaller E2 proteins (8, 26). However, additional analyses of E2 regulation have suggested that in some circumstances the full-length papillomavirus E2 proteins can also repress promoter activity. In transfected human epithelial cells, both the full-length BPV-1 E2 and HPV-18 E2 proteins can repress the basal activity of the HPV-18 transforming gene promoter, suggesting another mechanism of E2-mediated promoter regulation (5, 52).

The studies presented here were designed to examine the E2 regulation of the HPV-16 P_{97} promoter activity in primary human genital keratinocytes and to compare the E2 regulation of the P_{97} promoter with that of the analogous HPV-18 P_{105} promoter. In transiently transfected keratinocytes, the BPV-1 E2 gene product repressed the P_{97} promoter of HPV-16, and mutational analysis revealed that this repression was mediated through the $ACCN_6GGT$ E2-binding sites proximal to the promoter. Similar mutations of the analogous HPV-18 P_{105} promoter region revealed that E2-mediated repression occurred through the E2-binding site proximal to that promoter. We propose that E2 occupation of proximal E2-binding sites upstream of each promoter results in the negative regulation of transcription of the E6 and E7 genes, possibly by preventing the binding of transcription factors in these regions.

MATERIALS AND METHODS

Cell culture. Human keratinocytes were maintained as previously described (39). Cells were kept in KGM medium (Clonetics, San Diego, Calif.) and passaged by trypsinization followed by centrifugation through Dulbecco modified Eagle medium with 10% fetal bovine serum for trypsin inactivation. Pelleted cells were suspended in KGM medium and incubated at 37°C with 5 to 10% CO_2. C33 cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Cell transfections. Human keratinocytes were transfected in 100-mm tissue culture dishes with lipofectin (Bethesda Research Laboratories, Inc.) by using a modification of manufacturer specifications. For each transfection, a total of 5 μg of DNA (2.5 μg of chloramphenicol acetyltransferase [CAT] reporter plasmid DNA and 2.5 μg of E2 expression vector DNA or control plasmid DNA) was diluted in 50 μl of water and added to a polystyrene tube containing 1 ml of KGM. Lipofectin (50 μl) was added to the tube, and the contents were mixed gently and incubated at room temperature for 15 min. The DNA-lipofectin mixture was added to the keratinocytes, and the total amount of medium in each dish was brought to 2 ml. The cells were incubated for 2 h at 37°C, with rocking every 10 min. An additional 3 ml of medium was then added to each dish, and the cells were incubated for an additional 6 to 13 h prior to the addition of fresh KGM medium. Cells were harvested 48 h after transfection for CAT analyses. C33 cells were transfected by standard calcium phosphate techniques (53) by using 4 μg of CAT reporter plasmid, 2 μg of E2 expression plasmid DNA or control plasmid DNA, and 0.5 μg of a β-galactosidase plasmid (pRSV-β-gal). The following day, fresh medium was added to transfected cells prior to an additional 24-h incubation. Cellular extracts were prepared and assayed for CAT and β-galactosidase activities as previously described (51).

CAT assays. Transfected keratinocytes were prepared for CAT analysis 48 h after lipofection by using a modification of the procedure described by Gorman et al. (18). Cells were rinsed thoroughly with phosphate-buffered saline and then incubated for 5 min in 1 ml of buffer (0.04 M Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.15 M NaCl). Cells were collected by scraping, pelleted, and then disrupted by three freeze-thaw cycles. Lysates were collected after microcentrifugation, and protein content was determined by using Bio-Rad standards (Bio-Rad Laboratories). Typically, 100 μg of total protein was used for each CAT assay. For C33 cell lysates, one-fifth of the total cellular lysate (60-mm dishes) was used for CAT assays, and CAT activities were standardized by β-galactosidase activities. Cell lysates were incubated at 37°C with [^{14}C]chloramphenicol–4 mM acetyl coenzyme A in 0.25 M Tris hydrochloride (pH 7.8) for 30 min (C33 lysates) or 2 h (keratinocyte lysates). The acetylation products were separated by ascending thin-layer chromotography. Percent acetylation was determined by quantitation of counts in acetylated and unacetylated spots.

Recombinant DNAs. The LCR of HPV-16, cloned into a pTZ19 vector, was kindly provided by Karl Münger. The LCR had been modified by isolating the *Pst*I-*Hpa*II fragment of HPV-16 (nucleotides [nt] 7007 to 57), regenerating nt 58 to 103 with oligonucleotides, and adding additional nucleotides at the 3′ end of the fragment to generate enzyme sites convenient for cloning purposes. In generating the fragment 3′ to nt 103, nt 104 of HPV-16 was changed from an A to a G, altering the previously existing ATG sequence at this site. The reconstructed LCR, containing a native *Pst*I site at the 5′ end and a *Bgl*II site at its 3′ end, was cloned into the *Pst*I-*Bam*HI site of pTZ19 to generate the plasmid p1237. To isolate the LCR fragment used in the studies described here, the LCR was excised from p1237 by using *Hin*dIII, which cuts in the polylinker just upstream of the LCR insert, and *Bgl*II, which cuts at a site generated downstream from the LCR sequences. The *Hin*dIII site at the 5′ end of the LCR fragment was filled in with Klenow fragment, and *Bgl*II linkers were ligated to the ends of the fragment to generate flanking *Bgl*II sites. The LCR fragment was inserted into the *Bgl*II site of the CAT vectors $pA_{10}CAT2$ or $pA_{10}CAT3M$. $pA_{10}CAT2$, a derivative of pSV2CAT (18), contains the CAT gene driven by the enhancer-deleted simian virus 40 (SV40) promoter (24). $pA_{10}CAT3M$ contains the CAT gene but lacks both an enhancer and a promoter (23). pSV2CAT contains the CAT gene driven by the SV40 enhancer-promoter (18). p1318 is a human β-actin promoter vector (33); p770 is an SV40 promoter vector (35). The plasmid pC59 contains an unspliced BPV-1 fragment from nt 2360 to 4203, encoding the full-length BPV-1 E2 gene product, downstream of the SV40 early promoter (54). p1842 was constructed by the insertion of the small *Bam*HI fragment of the HPV-18 genome (nt 6929 to 119) into a unique *Bgl*II site upstream of the CAT gene in a derivative CAT expression vector lacking an SV40 enhancer or promoter. The *Bgl*II site in this vector had been inserted in the *Sal*I site just upstream of the unique *Hin*dIII site proximal to the CAT coding region.

Oligonucleotide reconstruction. For HPV-16, the 3′ region of the LCR from the *Hph*I site at nt 7773 to the *Bgl*II site downstream of the LCR in p1237 was generated by using the oligonucleotide synthesis method described by Grundström et al. (19) and modified by Spalholz et al. (48). Synthetic oligonucleotides were phosphorylated, hybridized to their complementary strands, and then ligated to a linearized

pUC19 vector for subsequent sequencing. Reconstructed fragments were used to replace their wild-type counterparts by ligation into the original LCR construct. For HPV-18, the p1842 construct was modified by destroying the *SalI* sites flanking the HPV-18 fragment. A set of 12 oligonucleotides was designed to replace a 220-nt region just upstream of the CAT sequence, from a unique *AccI* site at nt 7767 to position 119 of the HPV-18 genome. Reconstructed HPV-16 and HPV-18 LCRs were confirmed by sequence analysis.

RNA extraction and primer extensions. RNA was extracted from transfected C33 cells 40 h after transfection by the guanidinium lysis method of Chirgwin et al. (7) with the modifications described by Phelps and Howley (35). Total RNA (10 μg) was precipitated for analysis by primer extensions. A 20-base oligonucleotide complementary to the 5′ region of the CAT gene (5′-TTGGGATATATCAAC GGTGG-3′) was labeled at its 5′ end for use as a primer, and reactions were done as previously described (35).

Gel retardations. The overlapping ends of complementary synthetic oligonucleotides were filled in with [^{32}P]deoxynucleoside triphosphates using the Klenow fragment of *Escherichia coli* DNA polymerase. Gel retardations were performed by the method of Fried and Crothers (14), as modified by McBride et al. (29). The protein used for the binding assays was the COOH-terminal $E2_{290-410}$ protein (29), generated by in vitro transcription and translation and kindly provided by Alison McBride.

RESULTS

HPV-16 P_{97} promoter activity in primary human keratinocytes. The HPV-16 LCR contains elements which exhibit a certain specificity for keratinocyte factors (10) and are able to respond to the E2 gene products of papillomaviruses to modulate transcription (10, 35). In order to further examine the transcriptional regulation of the HPV-16 P_{97} promoter, a 1-kilobase fragment of the HPV-16 genome containing the cell-type-specific and E2-responsive enhancer regions of the LCR and the P_{97} promoter was inserted upstream of the CAT gene (Fig. 1 and 2A). Promoter activity in the presence or absence of BPV-1 E2 was then determined by CAT assays of transiently transfected primary human keratinocytes. Three types of CAT-expressing plasmids were used for these studies. The plasmid p16P was generated by cloning the LCR fragment (nt 7007 to 103) directly upstream of the CAT gene (Fig. 2A). In this construct, the LCR functions as both an enhancer and promoter of CAT gene transcription. Another plasmid, p16E, was constructed with the HPV-16 LCR (nt 7007 to 103) as an enhancer upstream of the SV40 early promoter, which directs CAT gene transcription. The third plasmid used was p863, which contains a truncated LCR (nt 7007 to 57) upstream of the SV40 early promoter and the CAT gene (35). The LCR in this construct does not contain the P_{97} promoter or the E2-binding site immediately upstream of the P_{97} promoter.

Primary human keratinocytes were transfected with equal amounts of p16P, p16E, or p863 DNA, and the activities of the promoters in the respective constructs were compared by CAT analysis of the lysates of transfected cells. The results with p16P (Fig. 2B) showed low basal activity of the P_{97} promoter in keratinocytes. This activity was similar to the basal activity of the LCR-SV40 promoter in p16E or p863 and was significantly lower than the activity of the SV40 enhancer-promoter construct (pSV2CAT). In contrast, basal P_{97} promoter activity could not be detected in a transfected human fibroblast line (WI38) or in a monkey kidney cell line

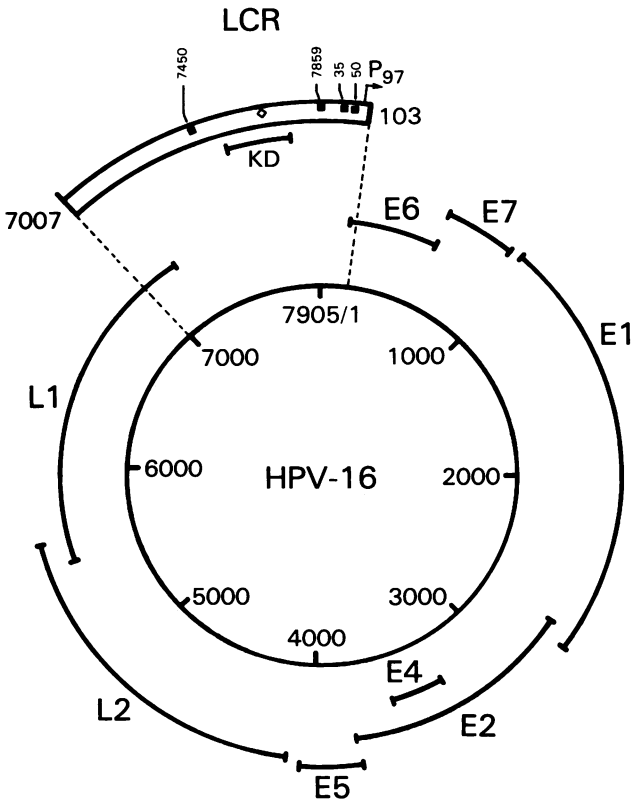

FIG. 1. Genomic map of HPV-16. The HPV-16 genome is drawn according to nucleotide sequence numbering (center of diagram) and location of early (E) and late (L) region ORFs. Consensus E2-binding sites (■) (2, 31), the glucocorticoid receptor binding element (◇) (16), and the keratinocyte-dependent enhancer (KD) (10) within the LCR are shown. E2-binding sites are numbered according to their nucleotide positions within the LCR. The LCR insert (nt 7007 to 103) illustrates the fragment of the genome used in these experiments. The P_{97} promoter is denoted by an arrow. Changes in the original sequence (43), including an additional E2-binding site at nt 7859 identified by sequence analysis and an additional nucleotide in the E1 ORF, generating an uninterrupted ORF (3), are shown.

(CV-1), although the SV40 promoter of pSV2CAT was active in both cell types (data not shown). These results are in general agreement with the observations of Cripe et al. (10), suggesting that the HPV-16 LCR contains a cell-type-specific enhancer which presumably can interact with specific factors for the activation of the P_{97} promoter. Basal P_{97} promoter activity in human keratinocytes was twofold higher than the negative control plasmid $pA_{10}CAT$, which contains the SV40 early promoter devoid of an enhancer element (data not shown). The absolute activity of the P_{97} promoter varied in different primary cell cultures, but the activity was reproducibly quite low and generally represented 1 to 5% of the activity of pSV2CAT.

E2-mediated repression of HPV-16 P_{97} promoter activity. Although the HPV-16 P_{97} promoter (p16P) and the SV40 early promoter linked to the HPV-16 LCR enhancer (p863 and p16E) had similar basal activities in human keratinocytes, each was affected differently by the full-length BPV-1 E2 gene product (Fig. 2B). The basal activity of the P_{97} promoter was repressed by E2 (p16P plus E2). In contrast, the SV40 promoter linked to either the complete or truncated LCR was transactivated by E2 (p16E plus E2 and p863 plus E2, respectively). The fact that BPV-1 E2 can activate the

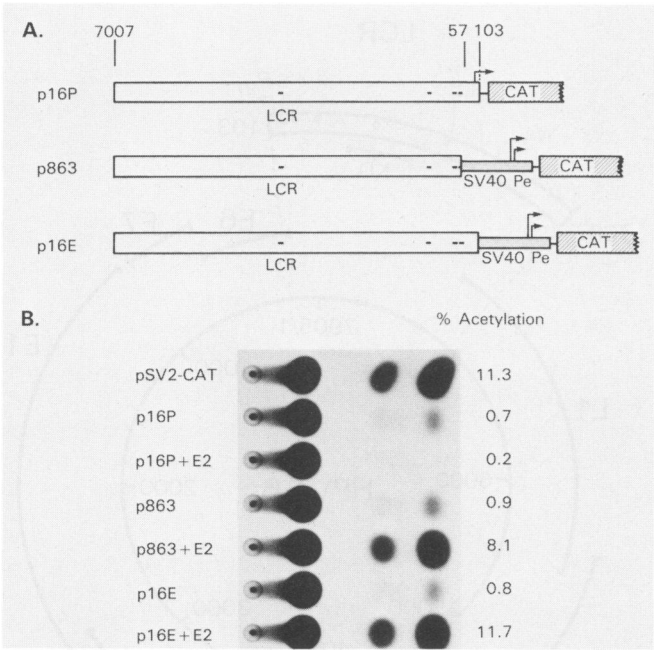

FIG. 2. P_{97} promoter activity in primary human keratinocytes. (A) Recombinant plasmid DNAs generated for transient transfections. The LCR (nt 7007 to 103) or a truncated LCR (nt 7007 to 57) was cloned upstream of the CAT gene or upstream of the SV40 early promoter (SV40 Pe) and the CAT gene to generate plasmids for transfections. The locations of the E2-binding sites within the LCR are shown (-). Arrows denote locations of promoters. (B) Autoradiograph of thin-layer chromatograph of CAT acetylation products. Primary human keratinocytes were transfected with 2.5 μg of p16P, p863, p16E, or pSV2CAT DNA together with 2.5 μg of either p1318 DNA (−E2 [control]) or pC59 DNA (+E2) for 8 h. Cells were harvested for CAT analysis 49 h after transfection. CAT assays were performed for 2 h at 37°C by using 100 μg of total cellular protein; the products were separated by ascending thin-layer chromotography and then excised and counted to determine the percentage of labeled acetylated chloramphenicol. pSV2CAT contains the SV40 enhancer and early promoter upstream of the CAT gene (18).

SV40 promoter in p863 and in p16E and can repress the P_{97} promoter in p16P indicates that E2 can differentially regulate the two promoters.

The experiments presented here show the repression of the HPV-16 P_{97} promoter by the heterologous BPV-1 E2 gene product. Additional experiments indicated that the HPV-16 E2 gene product was also capable of repressing the P_{97} promoter but to a lesser extent than BPV-1 E2 (data not shown). Similar results have been described for the repression of the HPV-18 and HPV-11 promoters by their homologous E2 gene products compared with the products of BPV-1 E2 (5, 6, 52). These HPV E2 gene products are also less efficient than BPV-1 E2 in their abilities to transactivate heterologous promoters linked to E2-dependent enhancer elements (6, 35, 52). In the case of HPV-16, the difference in this modulation may be due, at least in part, to the lower affinity of HPV-16 E2 for $ACCN_6GGT$ motifs in the HPV-16 LCR, compared with that of BPV-1 E2 (4a). We therefore used the BPV-1 E2 gene product to characterize the role of the individual E2 motifs within the HPV-16 LCR in the modulation of P_{97} promoter activity.

Analysis of the binding capacity of wild-type and mutant HPV-16 E2-binding sites. In order to determine the importance of E2-binding sites upstream of P_{97} for E2-mediated promoter regulation, each of the three motifs proximal to the promoter was mutated individually or in combination and reconstructed in the viral LCR. Double-stranded oligonucleotides containing wild-type or mutated forms of the E2-binding sites located at nt 7859, 35, and 50 within the HPV-16 LCR were synthesized to generate the motifs shown in Fig. 3A. To determine the binding efficiency of E2 protein to each site, the double-stranded radiolabeled oligonucleotide pairs were incubated with $E2_{290-410}$, an in vitro translated carboxy-terminal BPV-1 E2 protein capable of specific DNA binding (29). Protein-DNA complexes were detected on neutral polyacrylamide gels. The results show that each of the oligonucleotide pairs containing a wild-type copy of an E2 motif from the HPV-16 LCR (wt 7859, 35, and 50) could bind the BPV-1 E2 protein, although the nt 7859 motif had a much lower affinity for the protein than either the nt 35 or the nt 50 motif (Fig. 3B). In contrast, each of the oligonucleotide pairs containing a mutated motif (mt 7859, 35 or 50) did not bind E2 (Fig. 3C). These results support previous analyses of E2-binding-site specificity (2, 27, 31) and correlate the binding site mutations shown with a loss of E2-binding capacity at those sites.

***cis* elements involved in E2-mediated P_{97} promoter modulation.** The binding of E2 protein to $ACCN_6GGT$ motifs proximal to the HPV-16 P_{97} promoter may decrease promoter activity by the steric hindrance or displacememt of critical transcription factors, as postulated for the HPV-18 P_{105} promoter (5, 15, 52). One or more E2-binding sites may be involved in this type of repression, judging from the proximity of three different E2 motifs (nt 7859, 35, and 50; Fig. 1 and 2) to the promoter. In order to determine the role of the individual E2 motifs in E2-mediated promoter regulation, a 200-base-pair region of the HPV-16 LCR, reconstructed by the use of the synthetic oligonucleotides and containing wild-type or mutated E2-binding sites, was substituted in the plasmid p16P, resulting in the replacement of one, two, or three wild-type motifs with their mutant counterparts (designated motifs 1, 2, or 3, in order of their proximity to the P_{97} promoter; Fig. 4). The LCR sequences upstream of the nt 7859 E2 motif were left unchanged, leaving an intact E2 motif (designated motif 4) at nt 7450 in each of the constructs.

Plasmids containing wild-type or mutated LCRs were transfected into primary human keratinocytes, and the strength of the promoter in each construct in the absence or presence of BPV-1 E2 was determined by CAT analysis. The results (Fig. 4) show the importance of an intact E2-binding site at nt 50 for E2 repression of the P_{97} promoter. The addition of E2 to either the original wild-type plasmid (p16P) or the wild-type-reconstructed plasmid (p16P-4321) resulted in decreased basal promoter activity. In contrast, the mutation of the nt 50 motif (p16P-432X) led to a relief from E2 repression, regardless of the presence of wild-type E2 motifs at nt 35, 7859, and 7450, and resulted in an E2-mediated transactivation. The additional mutation of one or more of the motifs upstream of a mutated nt 50 motif also resulted in P_{97} promoter activation by E2 (p16P-4X2X, p16P-43XX, and p16P-4XXX). The strongest transactivation was noted in plasmids in which motifs 1 and 2 were mutated (p16P-43XX and p16P-4XXX). In contrast, none of the single or combined mutations in the upstream E2-binding sites (nt 7859 or 35) could impart E2 activation of the P_{97} promoter if the nt 50 motif was still intact (p16P-43X1, p16P-4X21, and p16P-4XX1). A mutation of the nt 7859 motif (p16P-4X21) resulted in a repression similar to that of a wild-type LCR (p16P and p16P-4321), and the additional mutation of the nt 35 motif

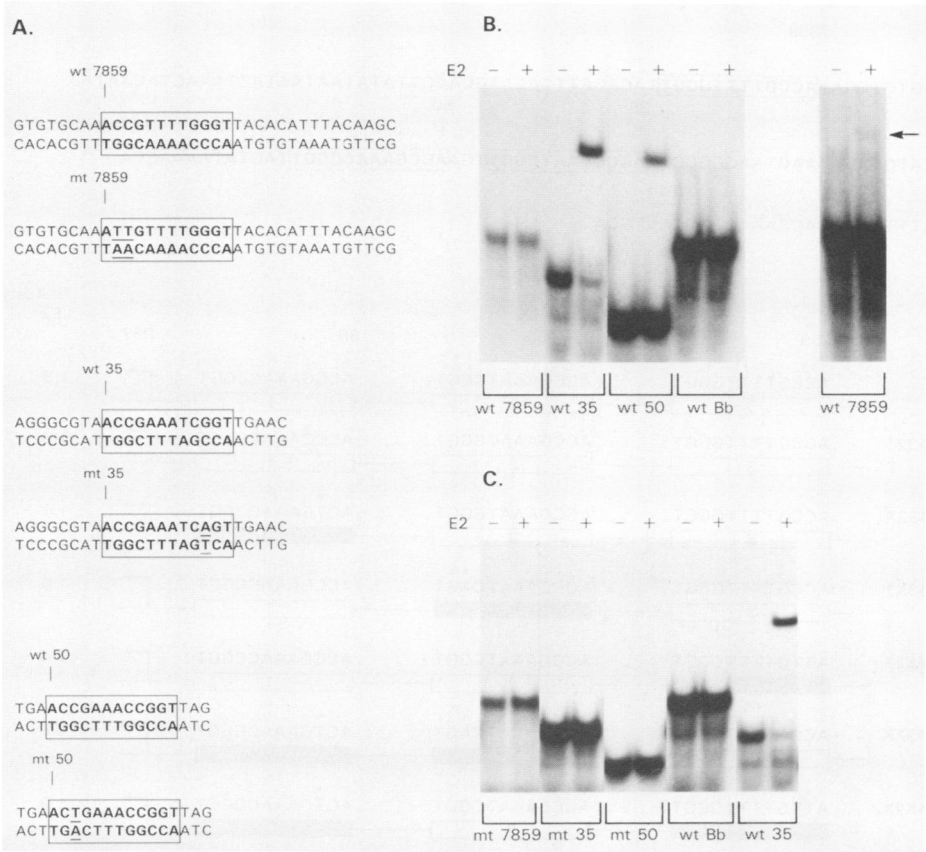

FIG. 3. Analysis of the E2-binding capacity of wild-type and mutant E2 motifs. (A) Double-stranded oligonucleotides containing wild-type (wt) and mutant (mt) E2 motifs corresponding to those at nt 7859, 35, and 50 of the HPV-16 genome were synthesized for use in in vitro binding analyses. Boxed sequences denote consensus $ACCN_6GGT$ motifs; mutated nucleotides are underlined. Oligonucleotides were labeled with [^{32}P]deoxynucleoside triphosphates, as described in the text. (B and C) Gel analyses of protein-DNA complexes. (B) Nucleotide fragments containing a wild-type E2-binding site (wt 7859, 35, or 50) or lacking an E2 site (wt Bb) were incubated with rabbit reticulocyte lysate (−) or lysate containing $E2_{290-410}$, an in vitro-translated BPV-1 carboxy-terminal protein (+). Protein-DNA complexes were analyzed on a 6% neutral acrylamide gel and autoradiographed. Two lanes (insert at right) were overexposed to show E2 binding to the wt 7959 motif; the arrow denotes the position of migration of the protein-DNA complex. (C) Fragments containing mt E2 motifs or wt (control) motifs were incubated in the presence of reticulocyte lysate without (−) or with (+) E2. Protein-DNA complexes were analyzed as in panel B. Bb is a DNA fragment lacking an E2-binding site, corresponding to HPV-16 nt 7824 to 7855. Numbers designate nucleotide positions in the HPV-16 genome.

(p16-4XX1) was ineffective in the relief of E2 repression. Although a mutated nt 35 motif (p16P-43X1) relieved E2 repression, this mutation was not sufficient for E2 transactivation. E2-mediated P_{97} promoter transactivation consequent to the single mutation of the nt 50 motif (p16P-432X) indicated the importance of this site for E2-mediated P_{97} promoter repression, while the stronger activation of the promoter subsequent to an additional mutation at nt 35 (p16P-43XX) indicated the added importance of this site for repression.

Comparison of HPV-16 and HPV-18 promoter activities. The HPV-18 P_{105} promoter is the functional equivalent of the HPV-16 P_{97} promoter (51). E2 repression of P_{105} promoter activity has been demonstrated in HeLa cells, in an adenocarcinoma cell line (SW13) (52), and in primary human keratinocytes (5). A comparison of the sequences upstream of the HPV-16 P_{97} promoter and the HPV-18 P_{105} promoter revealed a similar spatial arrangement of the four E2-binding sites, the TATAAAA boxes, and the start sites for transcription in the respective viral LCRs (Fig. 5). This suggested that both promoters could be regulated by similar mechanisms. In order to examine this possibility, the basal activities of the respective promoters and their modulation by E2 were compared in primary human keratinocytes.

The LCR of HPV-18 (nt 6929 to 119) was cloned directly upstream of the CAT gene to generate plasmid p1842. The P_{105} promoter in this construct directs CAT gene transcription (51), and the plasmid is therefore similar to the HPV-16 p16P plasmid. Primary human keratinocytes were transfected with an equal amount of p1842 or p16P DNA, and the activity of the promoter in each construct was compared. CAT analysis of transfected cell lysates revealed similar P_{97} and P_{105} basal activities in these cells and similar repression of those activities (three- to fourfold) by the BPV-1 E2 gene product (Table 1). These data indicate that the P_{105} promoter of HPV-18 and the P_{97} promoter of HPV-16 have similar cell-type-specific activities in primary human keratinocytes and that the promoters may be similarly regulated by E2.

E2 repression of the HPV-18 P_{105} promoter is mediated through the promoter-proximal E2-binding motif. Evidence for the E2 repression of basal HPV-16 P_{97} and HPV-18 P_{105} promoter activities and the proximity of the respective E2-binding motifs to other promoter elements is consistent with a model in which E2 interferes with the binding of

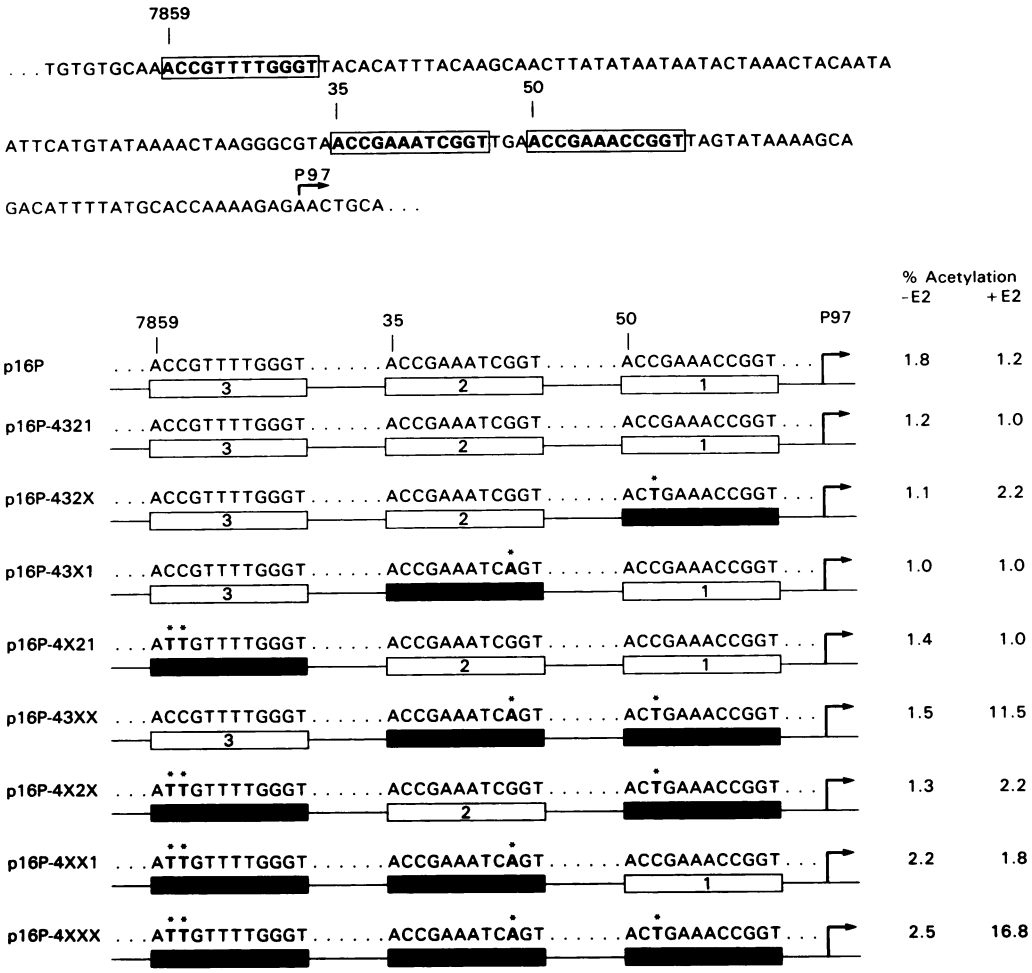

FIG. 4. Substitution of the E2-binding motifs in the LCR upstream of the P_{97} promoter. The HPV-16 fragment containing the three E2 consensus motifs and the promoter analyzed in these experiments is shown at the top of the figure. The boxed areas designate the E2 motifs, which are numbered according to their nucleotide positions in the genome; the arrow denotes the P_{97} promoter (P97). This fragment was reconstructed by oligonucleotide synthesis to generate the changes in the E2-binding sites shown at the bottom of the figure. Each respective fragment was substituted in the wild-type p16P vector where the LCR lies directly upstream of the CAT gene. Mutations in an E2-binding site are shown by bold letters with asterisks; all other nucleotides were kept identical to the wild-type LCR. Primary human keratinocytes were transfected for 15 h with 2.5 μg of each DNA, in addition to 2.5 μg of either p770 DNA (−E2) or pC59 DNA (+E2). Cell lysates were harvested 47 h after transfection, and 60 μg of total cellular protein was used for determination of CAT activity in a 2-h assay at 37°C. The relative basal activity of the P_{97} promoter varied slightly in experiments using different primary cell cultures. The differences in CAT activity for the plasmid constructs with the mutations of the individual E2-binding sites were consistent from culture to culture although all of the activities were reproducibly quite low. The results shown are the average of two experiments using the same primary keratinocyte cell population and are shown on the basis of percentage of pSV2CAT activity in these experiments. Open boxes signify consensus motifs capable of binding E2 in vitro; closed boxes signify those motifs which are mutated and no longer bind E2.

transcription factors when an E2-binding site proximal to each promoter is occupied by E2. In order to test this model, we first examined whether the E2 repression of the HPV-18 P_{105} promoter was mediated through the promoter-proximal E2 motif (nt 58). We then tested whether or not this repression was exhibited at a transcriptional level. Since the level of transcription from the HPV-18 P_{105} promoter was so low in primary human keratinocytes, these analyses were done in C33 cells, a human cervical carcinoma cell line which does not contain HPV-6, HPV-11, HPV-16, or HPV-18 DNAs (55) and does not exhibit E2 activity (F. Thierry, unpublished observations). The HPV-18 P_{105} promoter was similarly regulated by BPV-1 E2 in primary human keratinocytes and in C33 cells, but higher levels of transcription in C33 cells allowed an analysis of CAT RNA levels. CAT transcripts from the HPV-16 P_{97} promoter could not be detected in either primary human keratinocytes or C33 cells.

C33 cells were transfected with plasmids containing the LCR of HPV-18 upstream of the CAT gene. Plasmids containing a wild-type or mutated LCR, generated by oligonucleotide reconstruction, were analyzed for CAT activity in the presence and absence of E2 (Fig. 6). The plasmid p18P-4321 contains the wild-type HPV-18 LCR upstream of the CAT gene. The plasmid p18P-432X contains a mutation in the E2 motif immediately proximal to the P_{105} promoter, generating a site that does not bind E2 (data not shown). The third plasmid, p18P-4321t, contains the sequence GCCGACA substituted for the HPV-18 TATAAAA sequence at nt 73, creating a nonfunctional promoter (46). CAT analyses of lysates of transfected C33 cells showed the

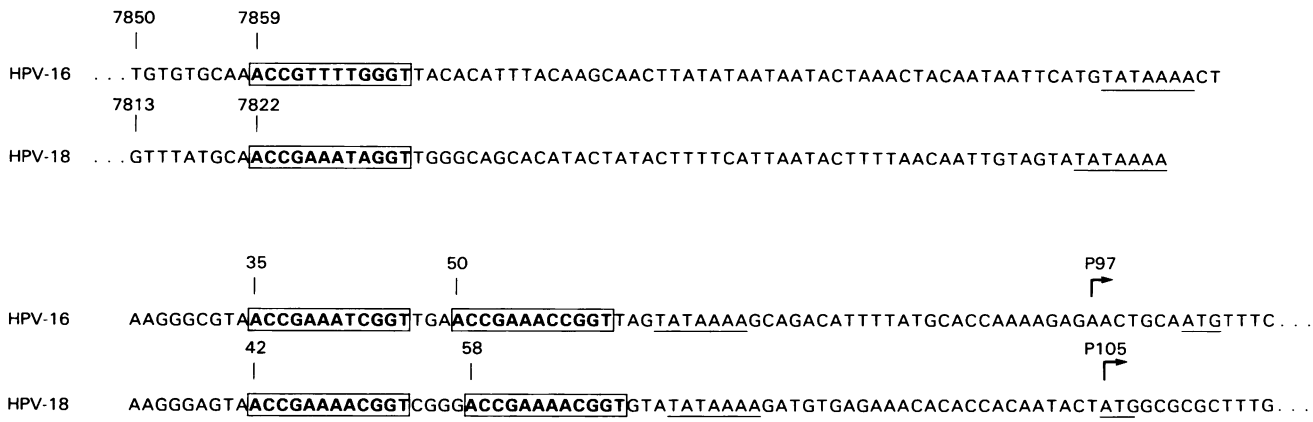

FIG. 5. Comparison of the HPV-16 and HPV-18 sequences in the vicinity of the respective P_{97} and P_{105} promoters. Sequences upstream of the HPV-16 and HPV-18 promoters are shown. E2-binding sites are boxed, and TATAAAA boxes are underlined. The promoters (P_{97} and P_{105}) are denoted by arrows; the first ATG downstream of the transcription start site in each genome is underlined.

importance of the integrity of the proximal E2-binding site for E2-mediated repression of the P_{105} promoter (Fig. 6). The basal activity of the P_{105} promoter was repressed ninefold in the presence of BPV-1 E2 in a wild-type LCR background (p18P-4321); the mutation of the nt 58 E2 motif relieved E2 repression (p18P-432X). Mutation of the HPV-18 TATAAAA box at nt 73 (p18P-1234t) resulted in a dramatic decrease in P_{105} activity either in the presence or absence of E2, confirming that P_{105} promoter activity was dependent on the integrity of the TATAAAA box. These results indicate that E2 repression of the P_{105} promoter is mediated through the E2 motif proximal to the promoter and suggest that E2 similarly regulates the HPV-18 P_{105} promoter and the HPV-16 P_{97} promoter.

E2 regulation of the HPV-18 promoter is transcriptional. To confirm that E2-mediated repression is a transcriptional effect, CAT RNA levels in C33 cells transfected with each of the plasmids shown in Fig. 6 were analyzed by primer extension. The results (Fig. 7) showed that the E2 modulation of CAT gene expression was indeed transcriptional. Although the E2 gene product only slightly repressed the basal level of CAT RNA transcription from the wild-type HPV-18 construct (p18P-4321), it strongly activated transcription from the construct containing a mutated E2 motif proximal to the P_{105} promoter (p18P-432X). Since the labeled primers were in great molar excess, the amount of the specific products detected by this assay (96- and 99-nt transcripts for P_{105}) was proportional to the amount of CAT RNA transcribed from P_{105}. The mutation of the P_{105} promoter (p18P-4321t) resulted in the absence of discernible CAT transcripts, suggesting that the wild-type promoter was responsible for the majority of the properly initiated transcripts detected in these experiments. These results are in good agreement with the data from the CAT analyses of transfected cell lysates and demonstrate that E2 modulation occurs at a transcriptional level.

TABLE 1. HPV-16 P_{97} and HPV-18 P_{105} promoter activity in primary human keratinocytes[a]

DNA	% Acetylation	
	−E2	+E2
p16P	0.8	0.2
p1842	1.1	0.4
pSV2CAT	13.8	ND

[a] Keratinocytes were transfected with 2.5 μg of p16P DNA, p1842 DNA, or pSV2CAT DNA, together with either 2.5 μg of p1318 DNA (−E2) or 2.5 μg of pC59 DNA (+E2). Cells were harvested 48 h after transfection, and 100 μg of total cellular protein was used for CAT assays in a 2-h reaction at 37°C. Products were separated by thin-layer chromatography; separated species were excised and counted to determine percentage of total labeled chloramphenicol acetylated. p16P contains the HPV-16 P_{97} promoter upstream of the CAT gene; p1842 contains the HPV-18 P_{105} promoter upstream of the CAT gene. ND, Not done.

DISCUSSION

The studies presented here indicate that the HPV-16 P_{97} promoter and the HPV-18 P_{105} promoter have comparable low levels of constitutive activity in primary human keratinocytes in the absence of any viral gene products. The BPV-1 E2 gene product repressed the basal activity of each promoter, and this repression was mediated through the E2-binding sites immediately upstream of the TATAAAA boxes of each of the promoters. In contrast, the HPV-16 LCR could serve to transactivate the SV40 promoter in the presence of E2. The HPV-16 P_{97}-CAT and HPV-18 P_{105}-CAT constructs in which the promoter-proximal E2-binding site was mutated (p16P-432X and p18P-432X) were also transactivated by E2. A comparison of the positions of the E2-binding sites upstream of each of these promoters shows the close proximity of E2-binding sites to the HPV-16 P_{97} and HPV-18 P_{105} promoters, in contrast to the more distant position of these sites from the SV40 promoter in the enhancer constructs p16E and p863 and in the HPV-16 and HPV-18 promoter constructs with mutated proximal E2-binding sites. These results suggest that E2-mediated activation or repression is dependent on the proximity of occupied E2-binding sites to the promoter.

The results presented here indicate that E2 regulation of the HPV-16 P_{97} and HPV-18 P_{105} promoters in human epithelial cells may involve both positive and negative modulation of the cell-type-specific basal activity of each promoter. The integrity of the E2-binding site immediately proximal to the HPV-16 P_{97} promoter and the HPV-18 P_{105} promoter is important for E2-mediated promoter repression; the mutation of each E2-binding site resulted in a promoter which could be transactivated by E2. From the data presented here, however, it appears that the full spectrum of the E2 modulation of the HPV-16 P_{97} and HPV-18 P_{105} promoters may involve the upstream E2 motifs as well. For HPV-

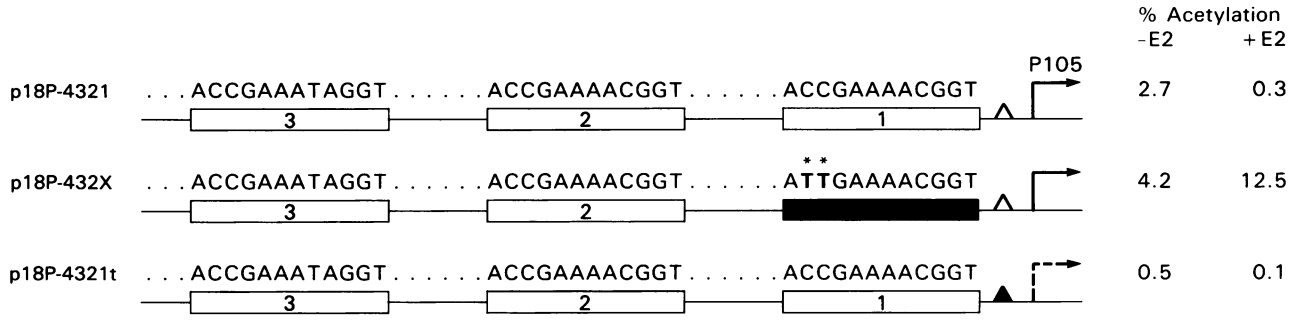

FIG. 6. CAT analyses of the HPV-18 P_{105} promoter activity in cervical carcinoma cells. The LCR of HPV-18 was generated by oligonucleotide synthesis to obtain mutations in the E2 motif proximal to the promoter or in the promoter itself. Mutated nucleotides are shown by bold letters with asterisks. Open triangles denote a wild-type TATAAAA box at nt 73; a closed triangle denotes a mutation of the TATAAAA box by a substitution with the sequence GCCGACA. Arrows denote the P_{105} promoter; the dotted arrow denotes a crippled promoter. Open boxes designate E2 sequences capable of binding E2; the closed box designates a mutated sequence no longer capable of binding E2. Promoter strength was determined by CAT enzyme activity of transfected C33 cell lysates, expressed as percent acetylation of total labeled chloramphenicol. Each plasmid DNA was transfected with pSV2neo DNA (−E2) or pC59 DNA (+E2).

16, there was little change in the level of P_{97} promoter activity following mutation of the nt 7859 motif (p16P-4X21), either in the presence or absence of E2, and mutation of both the nt 7859 and nt 50 motifs (p16P-4X2X) resulted in an E2 responsiveness similar to that of a mutation at nt 50 alone (p16P-432X). These results are not surprising considering the relatively low affinity of the nt 7859 motif for E2 (Fig. 3). However, since this site is conserved in the same position in HPV-16 and HPV-18, it seems unlikely that the motif has no physiological role for the virus; this site may function in a manner that is not revealed by these experiments. The role of the nt 35 motif for E2-mediated promoter regulation is also not obvious. In the context of a wild-type LCR, a mutation in the nt 35 motif (p16P-43X1) did not result in a significant change in the activity of the P_{97} promoter in the presence or absence of E2. However, this mutation in combination with a nt 50 motif mutation (p16P-43XX) resulted in the E2-mediated activation of the P_{97} promoter, suggesting that the nt 35 motif may also be involved in negative regulation of the promoter. Furthermore, the motifs at nt 7859 and 7450 might contribute together or individually in an E2-mediated enhancerlike fashion in the transactivation of the P_{97} promoter. The mutation of all three motifs within 150 nucleotides of the P_{97} promoter resulted in an E2-mediated promoter activa-

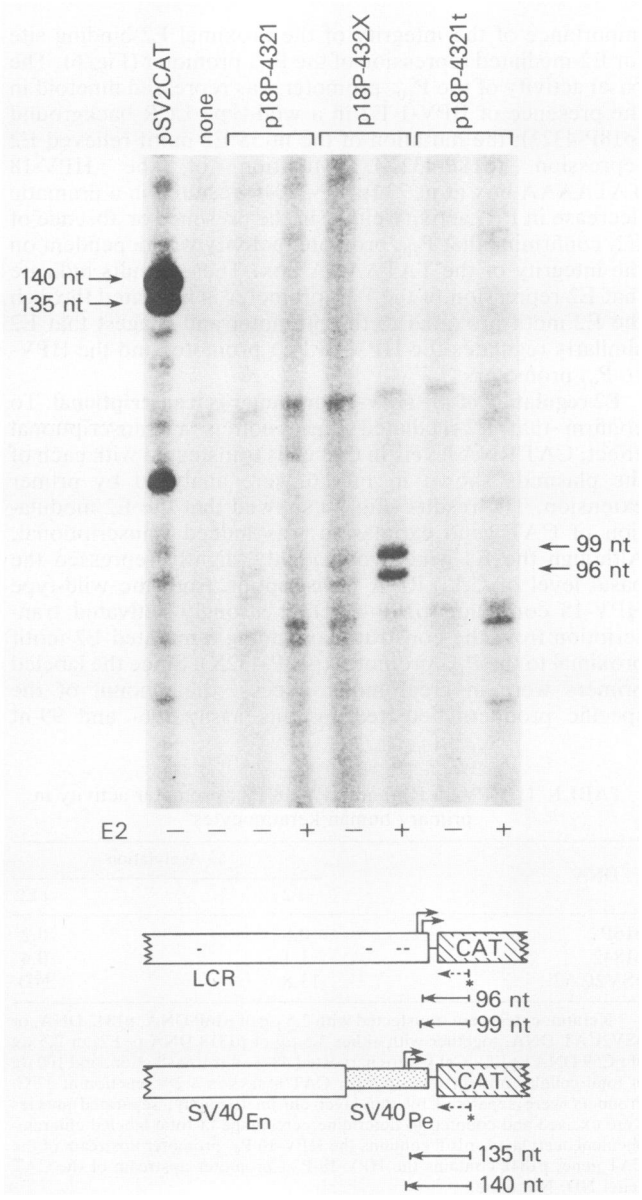

FIG. 7. Analysis of CAT transcripts from the HPV-18 P_{105} promoter by primer extension. C33 cells were transiently transfected with the LCR-CAT constructs shown in Fig. 6 in the presence (+) or absence (−) of the BPV-1 E2 gene product. Cells were harvested for RNA extraction and analysis 40 h after transfection. Total RNA (10 μg) from each transfection was hybridized to a 5′-end-labeled CAT primer (dotted arrow under the CAT gene). The primers were extended by using reverse transcriptase, and the products were analyzed on a 6% polyacrylamide gel and autoradiographed. Expected primer extension products are shown at the bottom of the figure. The migration positions of two species of transcripts derived from the HPV-18 P_{105} promoter (96 and 99 nt) and the SV40 early promoter (135 and 140 nt) are shown. Lanes are marked with the DNA used for each transfection with (+) and without (−) E2. Cells were transfected with pSV2CAT (contains the SV40 enhancer and early promoter upstream of the CAT gene), no DNA (none), a construct containing the wild-type HPV-18 LCR upstream of the CAT gene (p18P-4321), a construct containing an HPV-18 LCR with a proximal E2-binding site mutation (p18P-432X), or a construct containing a P_{105} promoter mutation (p18P-4321t). $SV40_{En}$, SV40 enhancer; $SV40_{Pe}$, SV40 early promoter.

tion. It is possible that some of this activity could be due in part to the general transactivation effect by E2, which is independent of DNA binding (20, 49). However, we cannot rule out the possibility of E2 activation dependent on *cis*-acting elements, since an intact E2-binding site was present at nt 7450 of the LCR and additional E2-binding sites exist within the CAT vector. A plasmid with mutations in all four HPV-16 LCR motifs was not tested in this study.

The experiments described here show the repression of the HPV-16 P_{97} promoter and the HPV-18 P_{105} promoter by the BPV-1 E2 gene product. These results are in disagreement with an observation made by Cripe et al. of HPV-16 P_{97} promoter activation by the BPV-1 E2 gene product (10). The reason for this discrepancy is not apparent; however, the repression of the HPV-16 P_{97} promoter described in our study is based on the results of multiple experiments and is in agreement with evidence for the repression of the equivalent promoters of HPV-11 and HPV-18 by BPV-1 E2 (5, 6, 52). Furthermore, the similarity in the organization of E2-binding sites in the control regions of these three genital-type-HPV genomes predicts that these promoters may be similarly modulated by BPV-1 E2.

In the experiments described here, the heterologous BPV-1 E2 product was used because of its greater efficiency at promoter modulation. However, to fully understand the role of E2 in the regulation of HPV-16 and HPV-18 promoter activities in vivo, the regulation by the homologous HPV E2 gene products will have to be determined. The relative affinities of the E2 proteins of each of these viruses for the E2 motifs in the viral genomes, as well as the relative activities of their transactivation domains, will undoubtedly contribute to the nature of the E2 interaction with their homologous LCRs and the subsequent regulation of transcription.

In the context of the LCR, the roles of the individual E2 motifs may be quite complex, resulting in a dynamic E2-mediated regulation of viral promoter activities. The level of activation or repression may be due in part to the affinity of E2 for the individual binding sites (32). In vitro binding analyses using cloned HPV-16 LCR fragments indicate that the motifs at nt 35, 50 and 7450 have a high affinity for BPV-1 E2, in contrast to the low affinity of the nt 7859 motif (Fig. 3) (4a). Differences in these affinities may serve to regulate the activity of the P_{97} promoter as well as the other putative promoters not yet mapped in the genome. The accessibility of the individual sites to E2 could also be involved in regulation of the promoter. Binding to the upstream E2-binding sites could result in transactivation of the promoter if the proximal E2-binding sites were not available for binding, possibly because of the presence of cellular transcription factors (15–17). In addition, E2 modulation of HPVs may involve E2 repressor products similar to those found for BPV-1 (8, 26), although at this time, no such factors have yet been described for HPV-16 or HPV-18.

In this paper, we have shown that the HPV-16 P_{97} and HPV-18 P_{105} promoters can be negatively regulated by E2. The model of E2 regulation presented here is consistent with a model in which there is a derepressed regulation of the P_{97} and P_{105} promoters after viral integration in the E1 or E2 genes with the consequent loss of E2 expression (3, 40, 41, 45). This integration event would result in changes in viral gene expression due to the disruption of the E2 gene and the subsequent loss of E2 transcriptional regulation. The loss of E2 could then be predicted to provide a selective advantage for cell growth due to the consequent deregulated expression of the viral E6 and E7 oncoproteins. Additionally or alternatively, a disruption of the E1 ORF may destroy the E1 functions necessary for extrachromosomal persistence and transcriptional regulation, assuming that the E1 functions are analogous to those of BPV-1 (25, 28, 38). An integration event that stably associates the viral genome with the cell would provide a selective growth advantage if viral DNA replication and genome partitioning were not efficient and faithful processes for these HPVs. Viral integration may provide the means for an interaction with host transcription factors that results in a latent viral existence. The LCRs of both HPV-16 and HPV-18 bind numerous cellular factors (15–17). Further analyses need to distinguish the importance of these interactions for HPV gene expression in primary epithelial cells and to determine whether there are differences in the types or strengths of these interactions during cellular differentiation. Such studies may provide insight into the mechanisms of cellular control of HPV-16 and HPV-18 gene expression.

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