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Inducible and Constitutive Enhancer Domains in the Noncoding Region of Human Papillomavirus Type 18

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The noncoding region of human papillomavirus type 18 (HPV-18) is shown to contain at least three enhancer elements. Two of these elements are responsive to papillomavirus-encoded *trans*-acting factors, and the third element functions as a constitutive enhancer, requiring only cellular factors for activity. The first enhancer (IE2) is located proximal to the E6 cap site and is responsive to papillomavirus E2 *trans*-activator. The second enhancer (IE6) is located approximately 500 base pairs upstream of the E6 cap site and is dependent upon the viral E6 gene product for function. A third enhancer (C) is located between 200 and 400 base pairs upstream of the E6 cap site and possesses a constitutive activity, requiring no HPV-18-encoded factors for function. The constitutive enhancer element exhibits some cell type preference for epithelial cell lines, but also functions in rodent fibroblast lines. Each of these enhancers manifests activity independent of the other elements and may reflect separate transcriptional control elements for different stages of the HPV-18 virus life cycle.

Papillomaviruses are small DNA viruses that are responsible for a wide range of benign tumors in humans and other animal species. Subtypes of the human papillomaviruses (HPV) are the presumed etiological agents of carcinomas of the cervix and penis (7, 11, 20). HPV types 16, 18, 31, 33, and 35 have been found in over 80% of all cervical tumors, suggesting that these viruses play a causative role in the development of these malignancies (7, 11, 19, 33). In the majority of lesions, some or all of the copies of the HPV genomes are found integrated into the host genome (7, 19, 24). Viral integration often interrupts the E1 or E2 open reading frame (ORF), with resultant disruption of E2 expression. In cervical tumor cell lines, such as HeLa and MS 751, in which the virus is integrated, only the E6 and E7 ORFs are expressed at high levels (2, 22, 25). In benign lesions, however, the virus is usually found in an episomal state with a larger spectrum of early gene expression (19). In both situations, a large portion of viral gene expression is controlled by sequences found in the noncoding region, analogous to the transcriptional regulation found in bovine papillomavirus type 1 (BPV-1).

The noncoding region (alternatively referred to as the long control region or upstream regulatory region) of BPV-1 has been shown to contain sequences for the regulation of viral replication (16, 17), plasmid maintenance (16, 17), and gene expression (13, 26, 27). HPV-16 and HPV-18 share significant organizational homology with BPV-1 (2, 8, 9), containing similar ORFs in analogous places on the virus (Fig. 1). The BPV-1 noncoding region contains a conditional enhancer element that is inducible in *trans* by the BPV-1 E2 ORF (21, 27). An analogous E2-inducible enhancer has also been identified in the noncoding region of HPV-16. This enhancer is responsive to both the BPV-1 E2 and the HPV-16 E2 proteins (23). In this study we have sought to identify transcriptional control sequences located in the noncoding region of HPV-18 and thus provide insight into the tissue tropism of this human tumor virus.

A promoter has previously been identified in the noncoding region of HPV-18 which is active in several cervical tumor lines, as well as in an adrenocortical carcinoma cell line (31). In addition, an enhancer element which is active in HeLa cells has also been localized to the HPV-18 noncoding region (29). In this study we have analyzed the noncoding region of HPV-18 in detail and identified three distinct enhancer elements which are capable of independent enhancer function. Two of these elements are inducible by HPV-18 gene products, and the third acts in a constitutive manner and does not require any HPV-18 gene products for its function. These three elements may be active during different stages of the HPV life cycle.

MATERIALS AND METHODS

Cell culture. African green monkey kidney CV-1 cells, mouse NIH 3T3 cells, rat-1 cells, human cervical tumor lines MS 751 and HeLa, and the cell line constructions described below were all maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 20 mg of gentamicin per liter. SCC-13 cells, a human squamous cell carcinoma cell line obtained from Elaine Fuchs, were maintained in E medium (32).

Plasmids. Plasmids pA10cat2 and pSV2cat are enhancer tester and control vectors that have been described previously (12, 15). Plasmid pC59 (obtained from P. Howley [27]) contains the BPV-1 E2 cDNA under the transcriptional control of the simian virus 40 (SV40) early promoter. Plasmids containing HPV-18 were obtained from H. zur Hausen.

The following chloramphenicol acetyltransferase (CAT) expression plasmids were derived from pA10cat2 and constructed during the course of this study. For p18catA, the 1.1-kilobase (kb) *Bam*HI fragment from HPV-18 containing the noncoding region and E6 cap site was cloned in the antisense orientation into the *Bgl*II site of pA10cat2. For p18BalA-1 through p18BalA-6, a series of deletions were generated in the noncoding region by BAL 31 digestion from the *Sal*I site of p18catA (18). Six of these deletions, designated p18BalA-1 through p18BalA-6, were shown by restriction enzyme analysis to contain deletions covering the entire 1.1-kb noncoding region. Several of these plasmids were

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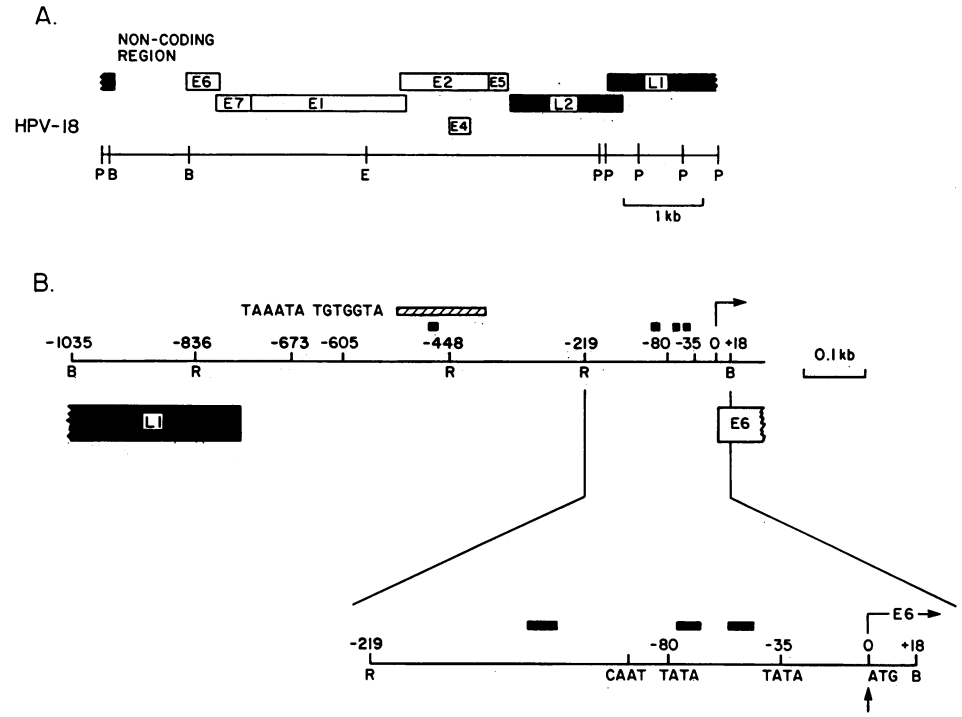

FIG. 1. (A) Genomic organization of the HPV-18 genome. ▭, Early region genes; ▬, late genes. The genomic organization is that of Cole and Danos (9). Restriction sites (B, *Bam*HI; E, *Eco*RI; P, *Pst*I) are indicated. (B) Expanded view of the noncoding region. The locations of putative E2-binding sites are indicated (▬), and an SV40 enhancer consensus sequence (TGTGGTA) is identified; a region of homology with the plasmid maintenance sequences of BPV-1 is indicated (▨), and a sequence (TAAATA) similar to that found in a BPV-1 promoter (28) thought to be involved in replication is also identified. A further expansion of the region from +18 to −219 bp is shown at the bottom of panel B, with an arrow indicating an early cap site mapped by Schneider-Gadicke et al. (24) and is designated nucleotide 0. Restriction sites (R, *Rsa*I; B, *Bam*HI) are indicated. The ATG start codon for E6 translation is also shown.

sequenced by standard dideoxy methods, and their deletion endpoints are indicated in Fig. 2. Plasmid p18BalA-1 contains a deletion of about 50 base pairs (bp) of noncoding region, removing the putative E6-E7 cap site. For p18catS, a *Sau*3A fragment containing the retained noncoding sequences of p18BalA-1 was inserted into the *Bgl*II site of pA10cat2 in the sense orientation. This construction resulted in deletion of the TATA box from the noncoding region. For p18BalS-1 through p18BalS-6, a series of BAL 31 deletions were generated from the *Sal*I site of p18catS through the noncoding region (Fig. 2). For p18CS and p18CA, a 229-bp *Rsa*I fragment (C fragment) from the noncoding region of HPV-18 was cloned in both orientations into the *Bgl*II site of pA10cat2 following the addition of *Bgl*II linkers (Fig. 3). For p18IE6A and p18IE6S, the 388-bp *Rsa*I fragment (IE6 fragment) from the HPV-18 noncoding region was cloned in both orientations into pA10cat2 at the *Bgl*II site following the addition of *Bgl*II linkers. For p18IE2A, p18IE2S, and pIE2 3′, a synthetic 50-bp oligonucleotide (GATCTGAGTGACGA AAACGGTCGGGACCGAAAACGGTGTATATAAAAGA) encompassing the two putative E2-binding sites (Fig. 3) was synthesized along with its complementary strand on an Applied Biosystems oligonucleotide synthesizer. The oligonucleotide was designed to contain *Bgl*II sites at either end. The two strands were annealed and then inserted into the *Bgl*II or *Bam*HI site of pA10cat2 to generate p18IE2A, p18IE2S, and pIE2 3′. For p18IE2M, a synthetic 37-bp oligonucleotide (GATCTGAGTGACCGAAAACGGTCGG GTATATAAAAGA) which contains a single E2-binding site was synthesized along with its complementary strand and cloned into the *Bgl*II site of pA10cat2.

Cell line construction. Several NIH 3T3 derivative lines were constructed by cotransfection of HPV containing plasmids with plasmids carrying either neomycin (pRSVneo) or mycophenolic acid (pSV2gpt) resistance genes. Individual drug-resistant clones were selected and expanded. A previously described cell line, p18PEpolyA:8 (abbreviated p18:8 [3]), is an anchorage-independent line that was derived by cotransfecting p18PEpolyA (Fig. 4) with pRSVneo and selecting for G418 resistance. The former plasmid contains a 3.5-kb *Pst*I-*Eco*RI fragment of HPV-18 and expresses the E6-E7 ORFs from the HPV-18 noncoding region. An SV40 late polyadenylation signal is used for transcription termination. The cell line p18PEBam:1 (M. Bedell et al., submitted for publication) was isolated following cotransfection of NIH 3T3 cells with RSVneo and plasmid p18PEBam, which contains a termination codon linker (*Hpa*I; Pharmacia Inc.) inserted into the E6 open reading frame at a *Bam*HI site of p18PEpolyA. This termination codon prevents translation of E6 but not E7. Three other NIH 3T3 lines, KS-10, KS-E6, and KS-E7, were derived after cotransfection of SV40 expression plasmids containing different ORFs of HPV-18 and either pRSVneo or pSV2gpt. The parental plasmid, KSV-10 (obtained from Pharmacia), contains the SV40 early promoter and T-antigen sequences for splicing and polyadenylation (Fig. 4). Fragments containing HPV ORFs were blunt ended with the Klenow fragment, ligated to *Bgl*II linkers, and cloned into the *Bgl*II site of plasmid KSV-10. The HPV-18 E6 and E7 ORFs, contained on 540-bp *Ava*II and 410-bp *Hae*II-*Sau*3A fragments, respectively, were cloned into KSV-10 to form pKS-18E6 and pKS-18E7, respectively. The cell lines derived after transfection are as

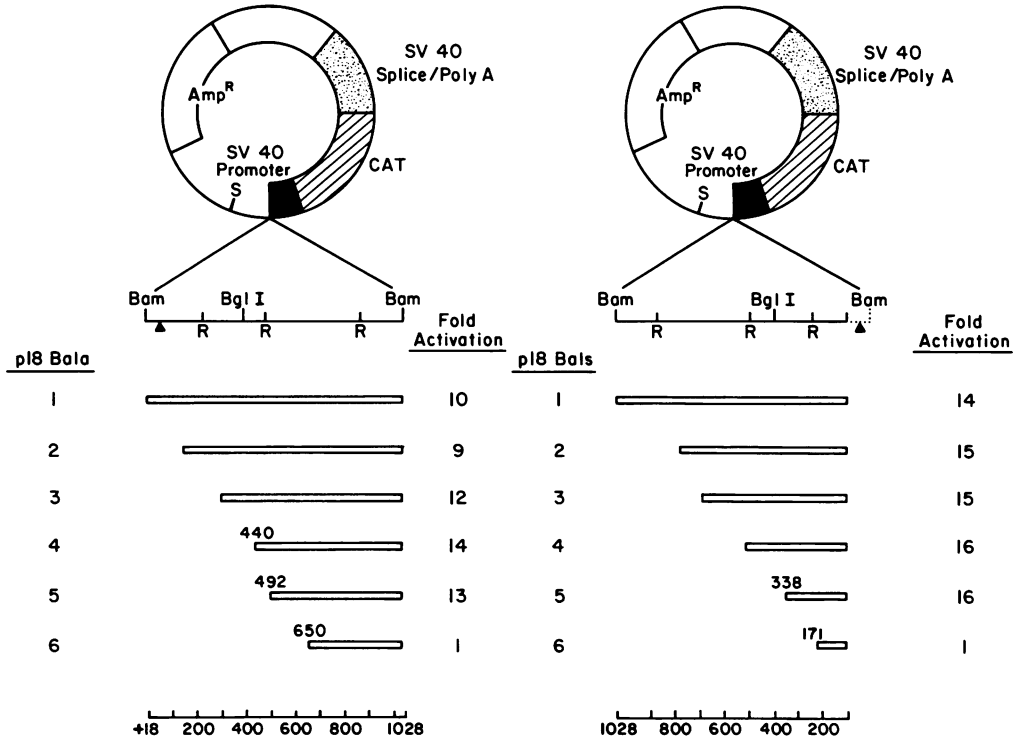

FIG. 2. Deletion analysis of the HPV-18 noncoding region. A series of BAL 31 deletions were constructed as described in Materials and Methods from the *SalI* site of p18catA and p18catS. These plasmids were transfected in duplicate into HeLa cells. In most cases two preparations of DNA were tested. The average level of activation above pA10cat2 levels is shown, with pA10cat2 levels normalized to 1.0. Several of the important clones were sequenced, and the nucleotide endpoints are shown. The location of the E6 cap site as mapped by Schnieder-Gadicke et al. (24) is indicated by a darkened triangle.

follows: KS-10 (KSV-10 control), KS-E6 (pKS-18E6), and KS-E7 (pKS-18E7).

Transfections and CAT assays. Transfections were done by calcium phosphate-mediated precipitation with a total of 30 μg of plasmid DNA per 100-mm dish. Precipitates were incubated overnight, and cells were harvested 24 h later. Cell extracts were prepared and CAT assays were performed as previously described (15). The typical incubation period of cell extracts with radioactive chloramphenicol was 30 min at 37°C. The amount of acetylation was determined by counting the acetylated and nonacetylated forms isolated by ascending thin-layer chromatography. All assays were performed in duplicate and repeated at least three times. Most assays were performed with at least two independent preparations of double-banded CsCl-purified plasmid DNA.

RESULTS

***Trans*-activation of HPV-18 transcription by E2.** We have used transient assays with plasmids containing CAT as a reporter gene to identify enhancer elements within the noncoding region of HPV-18. In these assays, activation of a heterologous SV40 promoter was used as the indicator of enhancer function. In all cases, CAT activity was normalized to that of the enhancerless plasmid, pA10cat2 (15). By using analogous methods, efficient *trans*-activation of the BPV-1, HPV-1, HPV-11, and HPV-16 noncoding regions by BPV-1 E2 has previously been demonstrated (14, 23, 27). To test whether the HPV-18 noncoding region is also *trans*-activated by BPV-1 E2, an E2-expressing plasmid (pC59) was cotransfected into CV-1 monkey kidney cells with p18catA, a plasmid into which the entire noncoding region of HPV-18 was cloned in the antisense orientation relative to the SV40 promoter (Fig. 3). In the absence of pC59, the CAT activity from p18catA was identical to that from the enhancerless pA10cat2 (Table 1). However, a low level of *trans*-activation (threefold) of the HPV-18 noncoding region by the BPV-1 E2 gene product was observed when pC59 was cotransfected with p18catA. A similar low level of activation was also observed for HeLa cells (Table 1), which are

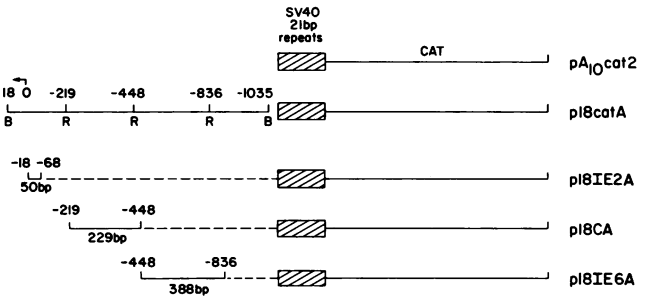

FIG. 3. Schematic illustrating constructs used to test for HPV-18 enhancer activity. The plasmid pA10cat2 contains the SV40 21-bp repeats driving expression of the CAT gene, but lacks any enhancer sequences. The remaining plasmids are derivatives of pA10cat2. p18catA contains a 1,053-bp *Bam*HI fragment encompassing the entire noncoding region of HPV-18 cloned in the antisense orientation upstream of the 21-bp repeats. The arrow indicates the location of the E6 cap site. p18IE2A contains a 50-bp synthetic oligonucleotide from −18 to −68 upstream of the E6 cap site. p18CA contains the 229-bp *Rsa*I fragment from −219 to −448 cloned in the antisense orientation. p18IE6A contains the 388-bp *Rsa*I fragment from −448 to −836 cloned in the antisense orientation.

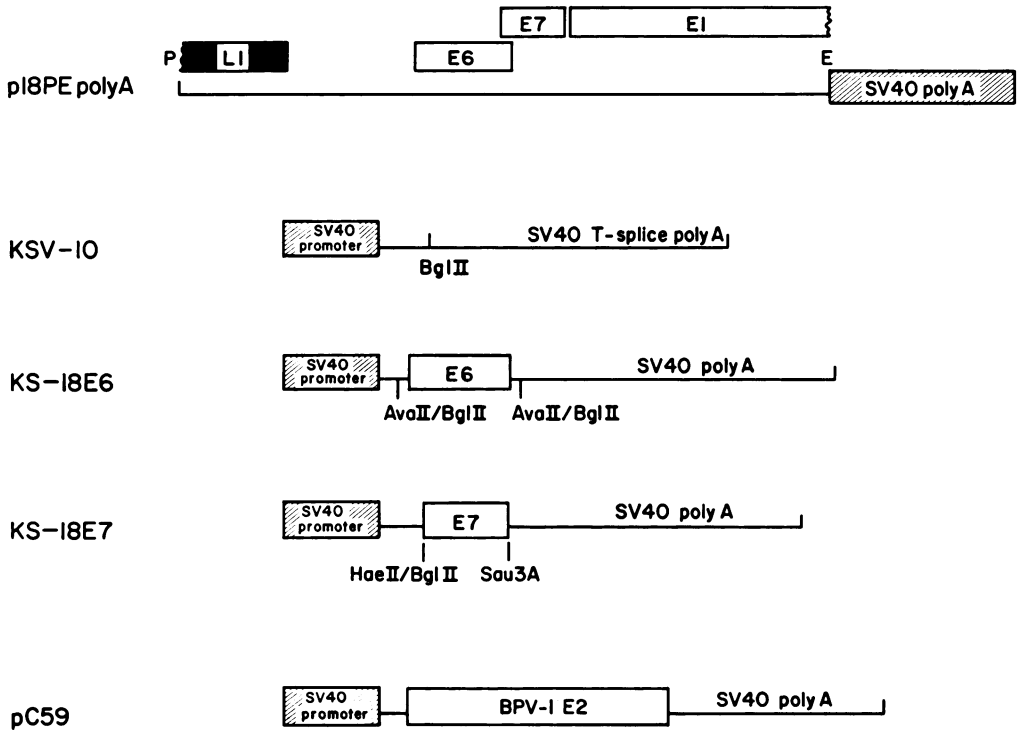

FIG. 4. Plasmids used to construct permanent NIH 3T3 derivative cell lines which express either or both of the E6 and E7 ORFs. Plasmid pC59 expresses the BPV-1 E2 gene product and was used only in transient assays. pPE18polyA contains the HPV-18 E6 and E7 ORFs driven by the autologous HPV-18 transcriptional regulatory sequences located in the noncoding region. KSV-10 is an SV40 expression vector which contains the SV40 early promoter, a *Bgl*II site for cloning, and T-antigen sequences for splicing and polyadenylation. pKS-18E6 contains the HPV-18 E6 gene. pKS-18E7 contains the HPV-18 E7 gene. pC59 contains a BPV-1 E2 cDNA under the control of the SV40 early promoter.

epithelial in origin and contain HPV-18 sequences. This low-level activation has been observed by others (14).

Like the HPV-16 noncoding region, the HPV-18 noncoding region contains the putative E2 reactive sequence (1), ACCGN4CGGT, repeated four times (9, 23). Two of these sites are located immediately adjacent to the putative early TATA box, and two other sites are located approximately 50 and 460 bp upstream of the E6 cap site (Fig. 1). To determine whether the two tandem E2-binding sites are sufficient for E2 *trans*-activation, a synthetic 50-bp oligonucleotide containing the two proximal E2-binding sites was cloned in both orientations into the *Bgl*II site of pA10cat2 (p18IE2A, p18IE2S). In contrast to the low induction levels with p18catA in CV-1 cells, p18IE2A and p18IE2S were induced over 40- and 70-fold, respectively, by the BPV-1 E2 gene product in this cell line. A comparable 50-fold level of activation is also observed in HeLa cells (Table 1). One possible explanation for the low level of E2 *trans*-activation observed with p18catA compared with p18IE2A and p18IE2S is the location of the E2-binding sites relative to the SV40 promoter sequences. In p18catA the E2-binding sites are located 1 kb from the SV40 promoter, raising the possibility that the activity of this element is distance dependent. To investigate this possibility, the 50-bp synthetic oligonucleotide was also cloned into the *Bam*HI site 3′ of the CAT gene in pA10cat2, placing the enhancer more than 2 kb downstream from the promoter (p18IE2 3′). The oligonucleotide activated expression 30-fold above background levels when located 3′ of the tester gene, demonstrating that these sequences can indeed function as an enhancer when located several kilobases from the promoter (Table 1). We have designated these 50-bp sequences IE2, for E2-inducible enhancer. The higher levels of activation observed with the 50-bp oligomer, as opposed to the entire noncoding region, suggest a complex regulatory mechanism, perhaps involving a negative regulatory element, for controlling viral gene expression.

To determine whether a single E2-binding site is sufficient for E2 activation, a synthetic 37-bp oligonucleotide containing one copy of the sequence ACCGAAAACGGT was cloned into pA10cat2 (p18IE2M) and cotransfected with pC59 into CV-1 and HeLa cells. No activation of p18IE2M by E2 was observed in either case (data not shown), suggesting that more than one E2-binding site is required for E2 activation. In addition, the 388-bp *Rsa*I fragment from the

TABLE 1. *trans*-Activation of HPV-18 enhancers by the BPV-1 E2 gene product

Plasmid	Relative CAT expression in following cells[a]:			
	CV-1		HeLa	
	−pC59	+pC59	−pC59	+pC59
pA10cat2	1	1	1	1
pSV2cat	122	91	300	300
p18catA	1	3	6	12
p18IE2A	1	72	1	50
p18IE2S	1	41	ND[b]	ND
p18IE2 3′	1	31	ND	ND

[a] Relative CAT expression is normalized to pA10cat2 levels. Assays were performed at 40 h posttransfection, and the average fold activation of several assays is shown. Tester plasmid (10 μg) was transfected with 20 μg of either pC59 or puc9 DNA.

[b] ND, Not determined.

TABLE 2. Cell type specificity of HPV-18 noncoding region enhancer function

Plasmid	Relative CAT expression in following cells[a]:		
	CV-1	HeLa	MS 751
pA10cat2	1	1	1
pSV2cat	92	191	50
p18catA	2.5	23	5
p18catS	2	36	ND[b]

[a] Relative CAT expression is normalized to pA10cat2 levels. Assays were performed at 40 h posttransfection, and the average fold activation of several assays is shown. A 30-μg amount of each tester plasmid was transfected.
[b] ND, Not determined.

noncoding region containing a single E2-binding site (Fig. 1) was cloned into the *Bgl*II site of pA10cat2 to generate p18IE6A and p18IE6S (Fig. 3). Cotransfection of these plasmids into CV-1 or HeLa cells also failed to show any E2-stimulated activity, further supporting the idea that two binding sites are required for E2 inducible function.

E2-independent enhancers in the HPV-18 noncoding region. The above data demonstrate that expression from the HPV-18 noncoding region can be activated by E2. However, in many cervical tumor lines, expression of the E2 ORF is disrupted by integration of the virus upstream of the E2 ORF. We sought to investigate the mechanism by which HPV-18 expression occurs in these cell lines. Plasmids p18catA and p18catS, containing the noncoding region in both orientations upstream of the SV40 early promoter of pA10cat2, were transfected into HeLa and MS 751 cell lines in which HPV-18 is integrated. In the absence of E2, the HPV-18 noncoding region is activated 23-fold in HeLa cells, 5-fold in MS 751 cells, and 2-fold in CV-1 cells (Table 2). The increase in transcriptional activity of p18catA in the experiments described in Tables 1 and 2 (6-fold and 23-fold activation, respectively) reflects differences in the amount of tester DNA transfected. The transcriptional activity of p18catA is not saturated at 10 μg of DNA, and the experiments in Table 1 were performed with 10 μg of tester DNA and 20 μg of carrier, whereas those in Table 2 involved 30 μg of tester plasmid.

HeLa and MS 751 cells contain HPV-18 transcripts exclusively from the E6 and E7 region of the virus (22, 25) and lack any detectable E2 transcripts. To determine whether virus expression in these cells is cell type specific or dependent upon HPV-18 gene products, we performed a series of experiments with an NIH 3T3 derivative cell line, 18:8 (see Materials and Methods), which is a permanent cell line that expresses the E6 and E7 ORFs from the autologous HPV-18 promoter (Fig. 4) (3). A consistent 3- to 5-fold activation of p18catA above pA10cat2 levels was observed in NIH 3T3 cells, and an additional activation to 10-fold was seen in 18:8 cells (Table 3). These results suggest that either E6 or E7 may act to *trans*-activate the HPV-18 noncoding region. If so, more than one type of enhancer element may be present. Surprisingly, cotransfection of p18catA with the E6-E7 expression plasmid p18PEpolyA resulted in only a twofold stimulation of HPV-18-directed expression (data not shown). Thus, *trans*-activation by the E6-E7 ORFs may require an established cellular state, since transient expression from the p18PEpolyA construct is insufficient for *trans*-activation.

To localize the E2-independent enhancer elements in the HPV-18 noncoding region, we made a series of BAL 31 deletions in both p18catS and p18catA (Fig. 2). Six deletions from each parent plasmid were isolated and assayed for CAT activity in HeLa cells. Deletions from the E6 cap site to nucleotide −492 (the E6 cap site is designated nucleotide 0) retained enhancer activity, but activity was abolished when sequences between −492 and −650 were deleted (Fig. 2). Deletions from the opposite end of the noncoding region retained enhancer function to nucleotide −338, but lost function at nucleotide −171 (Fig. 2). Plasmids p18BalA-1 through p18BalA-6 were also transfected into E6-E7-expressing 18:8 cells with similar results (data not shown). Although deletions from the early side of the noncoding region localize an enhancer between −650 and −492, deletions from the other end of the noncoding region (p18catS) localize an enhancer between −338 and −170. These results suggest that instead of a single enhancer, multiple or redundant E2-independent enhancers may be present. The presence of multiple enhancers in the noncoding region requires a more detailed analysis to separate constitutive from inducible activities.

Identification of constitutive and E6-inducible enhancers. In an attempt to separate the constitutive from the inducible activity, we made a second series of constructs that contained smaller fragments of the HPV-18 noncoding region. Two adjoining *Rsa*I fragments, 229 and 388 bp in length (Fig. 3), were cloned separately into the *Bgl*II site of pA10cat2 to form p18CA, p18CS, p18IE6A, and P18IE6S. These fragments roughly correspond to the regions implicated as enhancers from the BAL 31 data. The plasmids were transfected into NIH 3T3, 18:8, and HeLa cells, and the activation levels measured are shown in Table 3. Plasmids p18CA and p18CS were consistently expressed at 4-fold-higher levels above pA10cat2 levels in NIH 3T3 cells. In contrast, in HeLa cells p18CA was expressed at levels 28-fold-higher than pA10cat2. To distinguish whether this higher activity in HeLa cells was due to the epithelial nature of these cells or an HPV-18 *trans*-activating protein, we performed experi-

TABLE 3. Constitutive and E6 inducible enhancer activity in various cell lines

Plasmid	Relative CAT expression in following cells[a]							
	NIH 3T3	18:8	KS-10	KS-E6	KS-E7	p18PEBam:1	HeLa	SCC-13
pA10cat2	1	1	1	1	1	1	1	1
pSV2cat	59	48	47	50	61	50	180	153
p18catA	4	8	5	9	4	5	23	ND[b]
p18IE6A	1	10	1	9	1	1	8	ND
p18CA	4	4	3	5	3	ND	28	29

[a] Relative CAT expression is normalized to pA10cat2 levels. Assays were performed at 40 h posttransfection, and the average activation of several assays is shown. In 18:8 and HeLa cells, the E6 and E7 ORFs are expressed from the HPV-18 promoter; in KS-10 cells, only the SV40 early promoter is present; in KS-E6 cells, the E6 ORF is expressed from the SV40 early promoter; in KS-E7 cells, the E7 ORF is expressed from the SV40 early promoter; in p18PEBam:1 cells, the E7 ORF is expressed from the HPV-18 promoter.
[b] ND, Not determined.

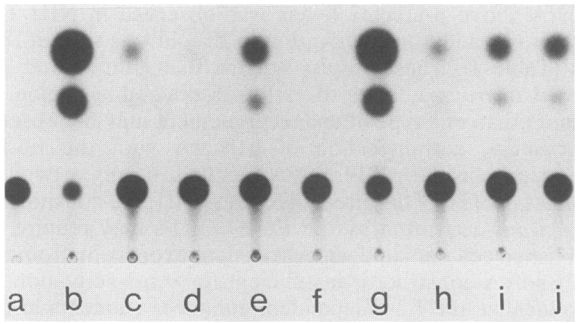

FIG. 5. Typical CAT assay showing relative CAT activities of the p18CA and p18IE6A plasmids in NIH 3T3 and 18:8 cells. Lanes a through e are NIH 3T3 transfectants, and lanes f through j are 18:8 transfectants. Lanes a and f, pA10cat2; lanes b and g, pSV2cat; lanes c and h, p18catA; lanes d and i, p18IE6A; lanes e and j, p18CA.

ments with SCC-13 cells, a human squamous cell carcinoma line which does not contain HPV sequences (data not shown). Transfection of SCC-13 cells with p18CA resulted in a 29-fold activation of expression, which is similar to the activation observed in HeLa cells. Since this 229-bp *Rsa*I fragment exhibits enhancer function in a variety of cells and is independent of HPV-18 gene products for function, we have designated this region C, for constitutive enhancer.

In contrast to the C enhancer, the 388-bp *Rsa*I fragment (p18IE6A) demonstrated no activation when transfected into NIH 3T3 cells (Table 3). However, when p18IE6A was transfected into 18:8 and HeLa cells, a 10- and 8-fold activation, respectively, was seen. A typical CAT assay autoradiogram for p18CA and p18IE6A transfections is shown in Fig. 5. These results suggest that the noncoding region of HPV-18 contains, in addition to the constitutive and the E2-inducible enhancers, another enhancer which is responsive to either the E6 or E7 ORF and which is located next to the L1 polyadenylation signal.

To help identify whether the E6 or the E7 gene product or both is responsible for the level of expression observed with plasmid p18IE6A, we constructed a series of permanent cell lines that individually express either of the ORFs. The E6 and E7 ORFs were separately cloned into an SV40 early expression vector, KSV-10, and cell lines containing pKS-18E6, pKS-18E7, and KSV-10 (Fig. 4) were isolated (see Materials and Methods). Briefly, KS-E6 expresses HPV-18 E6, KS-E7 expresses HPV-18 E7, and KS-10 expresses the parent expression vector KSV-10. Plasmid p18IE6A was tranfected into each of these lines, and CAT activity was determined (Table 3). p18IE6A was preferentially activated ninefold in the KS-E7 cell line, which expresses the HPV-18 E6 ORF, whereas no activation above pA10cat2 levels was observed in either the KS-10 or the KS-E7 line. To confirm that E6 provides a *trans*-acting function, p18IE6A was also transfected into a cell line, p18PEBam:1, which expresses the E6-E7 region from the autologous HPV-18 promoter, but contains a termination codon inserted into the E6 ORF. Consistent with the previous results, no activation of p18IE6A above backround was observed in the p18PEBam:1 cell line (Table 3), whereas in the 18:8 cell line, activation was 10-fold. Both p18PEBam:1 and 18:8 lines express comparable amounts of E6-E7 mRNA as determined by Northern (RNA) blot analysis (data not shown). The above studies identify the E6 gene product as a *trans*-activator of HPV-18 enhancer function, and we have designated the responsive element as IE6, for E6-inducible enhancer.

DISCUSSION

We have identified three distinct and independent enhancer elements in the noncoding region of HPV-18 (Fig. 6). Two of these elements are moderate activators of expression, of which one, referred to as C, appears to have a constitutive activity in a range of cell types including fibroblasts and human epithelial cells. A second enhancer, designated IE6 for E6-inducible enhancer, is activated in *trans* by the HPV-18 E6 gene product. A third element is proximal to the early cap site and is responsive to an E2 gene product. This element is referred to as IE2, for E2-inducible enhancer. The IE2 element is by far the strongest element, capable of being activated 50-fold by the BPV-1 E2 gene product. The effects of these enhancers does not appear to be additive, suggesting a complex interaction between the individual elements.

The IE2 enhancer element contains two putative E2-binding sites and is located between two Goldberg-Hogness boxes within 40 bp of the E6 cap site. In the presence of an E2 *trans*-acting protein from BPV-1, the HPV-18 IE2 is activated over 50-fold. The BPV-1 E2 *trans*-activator has previously been shown to be able to substitute for the HPV-16 E2 gene product and enhance expression of the HPV-16 noncoding region (23). This is not surprising, since the E2 ORF is well conserved in each of the papillomavirus genomes sequenced to date. In fact, the BPV-1 product is a stronger activator of HPV-16 expression than the HPV-16 E2 protein is. In the present studies, we have restricted ourselves to the BPV-1 E2 gene product, but experiments are in progress to confirm that the HPV-18 gene product has a similar effect.

The level of E2 activation observed when the entire HPV-18 noncoding region is used as an enhancer fragment (p18catA) in CV-1 or HeLa cells is only threefold. Similar results have been reported by Hirochika et al. (14). In contrast, when a 50-bp fragment containing only the two E2-binding sites was examined, a 50-fold activation by the E2 protein was observed. These results suggest that the noncoding region may contain a negative regulatory element which inhibits E2-inducible function in some cell types. The presence of other HPV-18 gene products or epithelial-specific proteins may overcome this inhibition.

Insight into the mechanism of E2 activation is provided by studies with plasmid p18IE2M, which contains a single putative E2 reactive site. Studies by Androphy et al. (1) have shown that a bacterially synthesized E2 protein binds to multiple copies of the sequence ACCGN4CGGT. We have

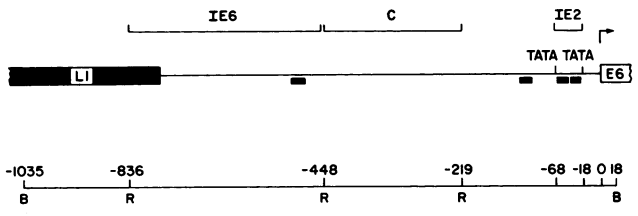

FIG. 6. Schematic illustration of the location of the three independent enhancer domains found in the noncoding region of HPV-18. The IE2 and IE6 regions are responsive to papillomavirus E2 and E6 *trans*-acting factors, respectively. The region designated C is a constitutive enhancer which is expressed in several cell types including rodent fibroblasts, but is most responsive in human epithelial cells. This element exhibits an apparent tissue preference rather than tissue exclusivity. ▬, Locations of putative E2-binding sites. B, *Bam*HI; R, *Rsa*I.

demonstrated that cotransfection of p18IE2M, a plasmid with a single E2-binding site, with pC59, an E2 expression plasmid, does not result in any appreciable activation above backround. In contrast, cotransfection of pC59 with plasmids containing two E2-binding sites (p18IE2A and p18IE2S) resulted in high-level activation. This suggests that at least two E2-binding sites are required for activation.

The virus exists as an episome in many benign lesions, but is found integrated into the host chromosome in most malignant lesions (7, 19, 24). The IE2 enhancer may be the predominant activator of viral gene expression during the episomal portion of the HPV-18 life cycle. However, as a result of integration, the viral genome is often disrupted such that E2 is no longer expressed. In the absence of E2 function, such as in the integrated state, expression of these genes is regulated differently than in the episomal state. Such a switch in the viral transcription pattern may be an important aspect in the transition from benign to malignant lesions. In fact, studies on HPV-18 transformation suggest that a subgenomic form of the virus expressing only the E6-E7 genes is an equivalent or better transforming agent than the entire viral genome (3).

In cervical tumor lines such as HeLa, the C and IE6 elements are the principal active enhancers, with C being the strongest. In addition, the C enhancer is sevenfold more active in the epithelial cell lines HeLa and SCC-13 than in rodent fibroblasts, suggesting that the constitutive enhancer may have a cell type preference for function. SCC-13 cells are an established tumor line, and the possibility exists that it is the transformed nature of these cells, rather than their epithelial character that is responsible for C enhancer function. Recently a consensus sequence has been identified in the enhancers of the bovine cytokeratin genes (AAPuCCAAA), which is also conserved in the upstream regulatory regions of the human and murine epidermal cytokeratin genes (6). This sequence is also present in the constitutive enhancer and may be responsible for the increase in enhancer activity observed in epithelial-derived cell lines.

In contrast to the C enhancer, the IE6 element is activated only in E6-containing cells. The IE6 enhancer, which contains an SV40 enhancer core sequence (TGTGGTA), maps upstream of the C element and is located at approximately 492 to 650 bp upstream of the E6 cap site. Although the promoter and cap site on which the IE6 enhancer acts have yet to be mapped, it is possible that the IE6 enhancer is not directly involved in early gene activation. Instead, its primary role may be in plasmid maintenance and replication. This is suggested by previous studies by Berg et al. (4, 5), who have shown that mutations in the BPV-1 E6 ORF dramatically reduce BPV-1 copy number in transformed C127 cells. In addition, a promoter that is postulated to play a role in plasmid replication (28) has recently been identified in the upstream region of the BPV-1 long control region. This BPV-1 promoter maps to the corresponding region of HPV-18 that we have identified as the E6-inducible enhancer. A sequence TAAATA is present in the BPV-1 promoter and is also found in the IE6 enhancer region of HPV-18 (Fig. 1). Whether HPV-18 contains a promoter in this region is currently under investigation. Since enhancer elements are often required to be adjacent to viral origins of replication (10), the IE6 element may be involved in papillomavirus episomal maintenance. This does not, however, rule out the possibility that the IE6 element acts as a transcriptional enhancer in the integrated state.

Recently, Thierry et al. (30) have examined the transcriptional activity of an HPV-18 promoter located near the E6 cap site in the noncoding region. Deletion analysis of the noncoding region indicated a 50% decrease in activity upon deletion of the 388-bp *Rsa*I fragment from the noncoding region (Fig. 1). Upon further deletion of the 229-bp *Rsa*I fragment, a total loss of transcriptional activity was seen. These results are consistent with our findings and suggest that there may be multiple enhancer elements within the noncoding region of HPV-18. Swift et al. (29) have identified a constitutive enhancer element within the 229-bp *Rsa*I fragment of HPV-18, in agreement with our identification of the C enhancer. However, no activity was reported for the HPV-18 388-bp *Rsa*I fragment in HeLa cells, which we have designated the IE6 enhancer. This discrepancy may be explained by a 2-bp substitution in the 388-bp *Rsa*I fragment of the isolate used by Swift et al. compared with the viral isolate used in this study (9).

In this study we have shown that the HPV-18 noncoding region contains at least three elements which function as enhancer elements in transient expression assays. The HPV-18 E6 gene product and an E2 gene product from BPV-1 can activate two of the enhancer elements present in this region, whereas the third enhancer is constitutive and may play the principal role in HPV-18 expression in cervical carcinomas. The E6-inducible element (IE6) may play a role in replication or plasmid maintenance, whereas the IE2 enhancer may play a major role in activating viral gene expression during the episomal portion of the virus life cycle.

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