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Activation of Human Papillomavirus Type 18 Gene Expression by Herpes Simplex Virus Type 1 Viral Transactivators and a Phorbol Ester

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Several viral *trans*-activators and a tumor promoter were examined for the ability to activate human papillomavirus type 18 (HPV-18) gene expression. A plasmid containing the HPV-18 noncoding region placed upstream of the chloramphenicol acetyltransferase reporter gene was cotransfected with different herpes simplex virus type 1 (HSV-1) genes into several cell lines. Both HSV-1 TIF and ICP0 activated HPV-18 expression; however, activation by TIF was observed only in epithelial cells, while ICP0 stimulated expression in a wide variety of cells. The element activated by both TIF and ICP0 was mapped to a 229-base-pair fragment which also contains an HPV-18 epithelial cell-preferred enhancer. The inclusion of a papillomavirus E2 *trans*-activator with TIF and ICP0 further increased HPV-18 expression. In contrast, the HSV-1 ICP4 and ICP27 genes, as well as the human T-cell lymphotropic virus type I and human immunodeficiency virus type 1 *tat* genes, were found to have no effect on HPV-18 expression. In transient assays, the addition of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) also activated HPV-18 expression. The region of HPV-18 activated by TPA was localized to a sequence which is homologous to other TPA-responsive elements.

Papillomaviruses are a group of small double-stranded DNA viruses that induce benign epithelial lesions. A small group of papillomaviruses is, however, involved in the etiology of malignant lesions (8, 13, 33). Human papillomavirus (HPV) types 16, 18, and 31 have been found in over 80% of cervical tumors, suggesting that these types play a causative role (13, 27, 36, 48). In malignant lesions, HPV DNA is integrated into the host genome, while in benign lesions the virus exists as a multicopy episome (37, 38). HPV integration often interrupts the E1 or E2 open reading frame (ORF), with resultant disruption of E2 expression (37, 39). In cervical tumor cell lines, most of the transcripts are derived from the E6-E7 region (4, 38), which contains a major transforming function (6, 23, 26).

Papillomaviruses contain multiple elements that act to regulate transcription during the viral life cycle. The papillomavirus E2 gene product is a strong *trans*-activator of bovine papillomavirus and human papillomavirus gene expression (11, 18, 41). The E2 protein activates expression by binding to the sequence ACCG(N)4CGGT, which is present four times in HPV-18 and six times in bovine papillomavirus type 1 (2, 10). In the absence of E2 expression, HPV-18 transcription is controlled by other elements, including a constitutive enhancer (Fig. 1) (11, 15, 42, 43). The constitutive element stimulates gene expression in several cell lines but shows a preference for cells of epithelial origin (15, 42).

Infection by HPV-16 and HPV-18 may not be sufficient for development of cervical cancer, and a second event may be required (45). Early studies have shown an association between the presence of both HPV and herpes simplex virus (HSV) genomes in cervical tumor cells (25, 34). These observations led us to examine whether the HSV TIF (*trans*-inducing factor), ICP0, ICP4, or ICP27 gene can alter the expression of HPV-18 (5, 14, 32). These HSV genes activate the expression of both HSV (30) and non-HSV (32) promoters. In this report, we show that the HSV TIF late (gamma) gene can activate expression of the HPV-18 noncoding region in epithelial cell lines, such as HeLa and SCC-13. In addition, the ICP0 immediate-early gene is capable of activating HPV-18 expression in a variety of cell lines, including HeLa, SCC-13, CV-1, and NIH 3T3.

Activation of HPV-18 expression is not limited to HSV viral cofactors and is also sensitive to chemical agents. We demonstrate that the HPV-18 promoter is activated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in transient assays performed in HepG2 cells. The DNA sequence responsible for TPA induction was localized to a 30-base-pair (bp) fragment of the HPV-18 noncoding region, which contains a TPA-responsive element (TRE). Furthermore, addition of TPA to a transformed NIH 3T3 cell line that expresses integrated copies of the HPV-18 E6-E7 region from the HPV-18 promoter resulted in an increase in expression, with a corresponding change in the morphologically transformed state of the cells.

MATERIALS AND METHODS

Cell culture. African green monkey kidney CV-1 cells, 18:8 cells (6), NIH 3T3 cells, and HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS) and 40 μg of gentamicin per ml. SCC-13 cells, a human squamous carcinoma cell line obtained from Elaine Fuchs, were maintained in E medium (47).

Plasmids and DNA preparation. Plasmids pA10CAT2, pTKCAT, and pSV2CAT are enhancer tester and control vectors (3, 16), while pCAT3m is a promoter tester plasmid that contains the *cat* gene but no promoter sequences (16).

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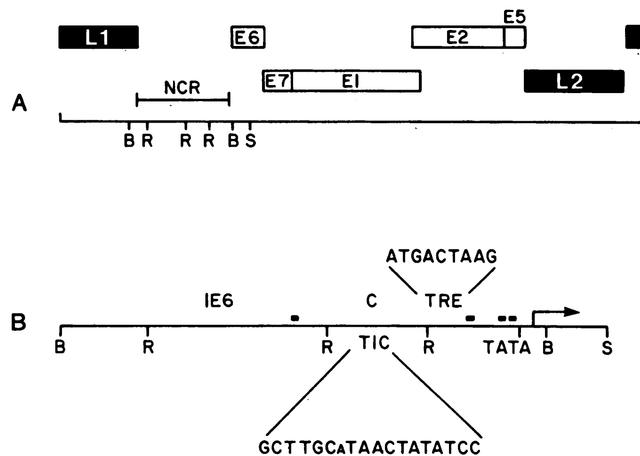

FIG. 1. (A) Genomic organization of HPV-18, with the early region designated by open boxes and the late region designated by solid boxes. The genomic organization is according to Cole and Danos (10). Restriction sites (B, *Bam*HI; R, *Rsa*I; S, *Sph*I) are indicated. NCR, Noncoding region. (B) Expanded view of the HPV-18 noncoding region, represented by a *Bam*HI-*Sph*I fragment. The locations of the putative E2-binding sites are indicated by small darkened rectangles. The previously identified E6 enhancer element (IE6) and the constitutive enhancer element (C) are identified (14). The E6-E7 promoter TATA box is shown, and an arrow indicates the transcription initiation site, which has been mapped by others (38, 42, 44). The sequence GCTTGCaTAACTATATCC represents the HPV-18 sequence with homology to the HSV-1 TIC, the *cis*-acting DNA sequence involved in TIF-mediated induction (24). The sequence ATGACTTAAG represents the HPV-18 sequence which contains 7 of the 8 bp of the TRE consensus sequence (3).

Plasmids p18CATa, p18IE6a, and p18Ca have been previously described (15). Briefly, these plasmids contain the entire HPV-18 noncoding region (p18CATa) or smaller subfragments of this region (p18IE6a and p18Ca) placed into the *Bgl*II site of pA10CAT2 (see Fig. 2). p18RBS was constructed by placing the 242-bp *Rsa*I-*Bam*HI fragment of the noncoding region into the *Bgl*II site of pA10CAT2. Plasmid p18CAT1221 consists of a 1,221-bp *Bam*HI-*Sph*I fragment containing the entire HPV-18 noncoding region cloned into the *Bgl*II site of pCAT3m (see Fig. 2). p18IETPA was constructed by synthesizing two complementary synthetic 30-bp oligonucleotides (GATCTATAATATGACTAAGCT GTGCATACA) which were annealed and placed into the *Bam*HI site of pTKCAT. pTKIE6 and pTKC were constructed by placing the 388-bp IE6 fragment and the 229-bp C fragment into the *Bam*HI site of pTKCAT, respectively (see Fig. 5). The HSV gene expression plasmids were a gift from Bernard Roizman and have been described in detail elsewhere (22). Briefly, pRB3522 expresses the HSV-1 TIF gene ORF contained in a *Sal*I F′-to-*Sal*I J′ fragment fused to a 216-bp *Bgl*II-*Sac*I fragment of the human metallothionein IIa promoter in pUC9. pRB420 expresses the ICP0 gene ORF from its natural promoter contained within a 4.35-kilobase *Sac*I-*Hpa*I fragment cloned into the *Sac*I site of pUC18. pRB419 expresses the HSV-1 ICP27 gene ORF from its natural promoter and is contained within a 2.4-kilobase *Bam*HI-*Sac*I fragment cloned into pUC13. pRB3611 expresses the HSV-1(F) ICP4 gene ORF from its natural promoter and contains the entire ICP4 gene ORF from −330 bp upstream of the transcription initiation site to 130 bp downstream of its polyadenylation sequences cloned in pUC18. Plasmid pc59 contains bovine papillomavirus type 1 (BPV-1) E2 cDNA under control of the simian virus 40 (SV40) early promoter and was a gift from P. Howley (41). The human immunodeficiency virus type 1 and human T-cell lymphotropic virus type I *tat* expression vectors (pSVETA and pHTLVtat-1, respectively) were obtained from Richard Gaynor (9, 35) and Rudy Pozzatti (28), respectively. p18PEpolyA was constructed by placing a 3.5-kilobase *Pst*I-*Eco*RI fragment of HPV-18 into the *Pst*I-*Eco*RI sites of pML2 (6).

Transfections and CAT assays. Transfections were by calcium phosphate-mediated precipitation with a total of 30 μg of DNA added to 10^6 cells per 100-mm-diameter dish. For cotransfection experiments, 10 μg of chloramphenicol acetyltransferase (CAT) tester DNA was transfected with 20 μg of carrier DNA (pUC9 or πSVx DNA, which gave identical results) or 20 μg of a *trans*-acting gene expression vector. Cells were incubated with precipitates overnight and harvested 24 h later. Cell extracts were prepared, and CAT assays were performed as previously described (16). The amount of acetylation was determined by counting the acetylated and nonacetylated forms separated by ascending thin-layer chromatography.

Transient assays involving TPA (Sigma Chemical Co.) (3) were performed following calcium phosphate-mediated precipitation with 4 μg of DNA added to HepG2 cells cultured in 0.5% FCS for 4 h, followed by glycerol shock. After 36 h, TPA was added to the cells at a concentration of 240 ng/ml, and cells were harvested 5 h later. CAT assays were performed in duplicate and repeated at least three times.

Addition of TPA to NIH 3T3 and 18:8 cells. NIH 3T3 and 18:8 cells were plated at a concentration of 10^6 cells per 100-mm-diameter plate in Dulbecco modified Eagle medium supplemented with 10% FCS and 40 μg of gentamicin per ml. The cells were allowed to attach, and 24 h later fresh medium was added. Control cells were cultured without TPA while two plates each of NIH 3T3 and 18:8 cells received 160 or 240 ng of TPA per ml.

The 18:8 cells were plated at a concentration of 10^6 cells per 100-mm-diameter plate and incubated with or without 240 μg of TPA per ml for 16 h. Total cellular RNA was isolated by the guanidium isothiocyanate method (6), and 20 μg was analyzed by Northern (RNA) blots.

RESULTS

***trans*-Activation of the HPV-18 promoter by HSV gene products.** Transient assays were performed with plasmids containing the CAT gene as a reporter gene to determine whether any HSV-1 gene products are capable of activating HPV-18 expression (16). In these assays, activation of the HPV-18 promoter, contained within a 1,221-bp *Bam*HI-*Sph*I fragment (p18CAT1221), was used as an indicator of gene expression (Fig. 2). The transcription initiation site for the HPV-18 E6-E7 promoter has been mapped to the region near the ATG of the E6 ORF (44). p18CAT1221 was cotransfected into CV-1 cells with either carrier DNA or the HSV-1 *trans*-acting genes TIF, ICP0, ICP4, and ICP27. In the presence of carrier DNA, the CAT activity induced by p18CAT1221 was identical to that seen with pCAT3m (Table 1), a promoterless CAT plasmid (Fig. 2). However, when p18CAT1221 was cotransfected with pRB420 (expressing the ICP0 gene), a 10-fold increase in expression was observed. No activation of p18CAT1221 was observed with pRB3522 (expressing the TIF gene), pRB3611 (expressing the ICP4 gene), or pRB419 (expressing the ICP27 gene) (Table 1).

These experiments were also performed in HeLa cells, a

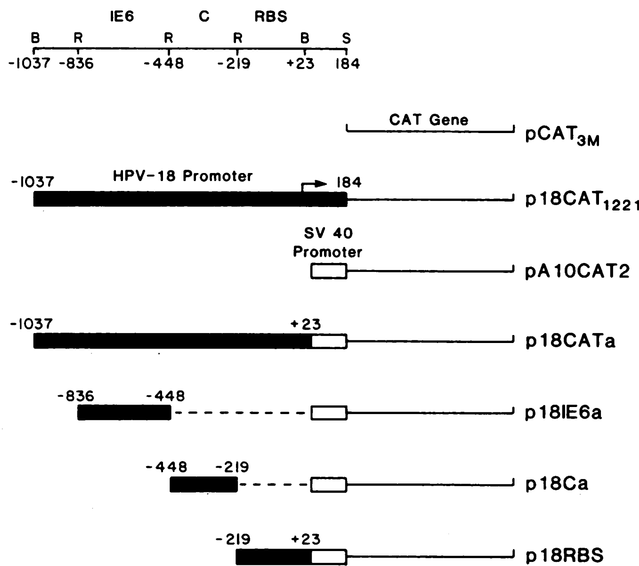

FIG. 2. Schematic diagram illustrating the constructs used to localize the TIF- and ICP0-responsive sequences in the HPV-18 noncoding region. Plasmid pCAT3m contains only the *cat* gene and was used to test for promoter activity. p18CAT1221 contains a 1,221-bp *Bam*HI-*Sph*I fragment cloned into the *Bgl*II site of pCAT3m from +184 to −1,037 bp relative to the E6-E7 transcription initiation site. Plasmid pA10CAT2 contains the SV40 21-bp repeats driving expression of the *cat* gene but lacks any enhancer sequences. Plasmids p18CATa, p18IE6a, p18Ca, and p18RBS are derivatives of the pA10CAT2 construct (14). p18CATa contains the 1,060-bp *Bam*HI fragment from −1,037 to 23 bp upstream of the E6-E7 transcription initiation start site. p18IE6a contains the 388-bp *Rsa*I fragment from −448 to −836 bp. p18Ca contains the 229-bp *Rsa*I fragment from −219 to −448 bp. p18RBS contains the 242-bp *Rsa*I-*Bam*HI fragment from +23 to −219 bp.

cervical tumor cell line that expresses the HPV-18 E6 and E7 ORFs (38). In the presence of carrier DNA, the CAT activity induced by p18CAT1221 was 16-fold higher than that seen with pCAT3m (Table 1). The activation of p18CAT1221 expression in these cells is due to an epithelial cell-perferred enhancer element (15, 43). However, when p18CAT1221 was cotransfected with pRB3522 (expressing TIF) expression increased to 68-fold, while the pRB420 (expressing ICP0) expression increased to 45-fold (Table 1). No activation of p18CAT1221 was observed with pRB3611 (expressing ICP4) or pRB419 (expressing ICP27). To determine whether

TABLE 1. *trans*-Activation of the HPV-18 promoter (p18CAT1221) by HSV-1 TIF, ICP0, ICP4, and ICP27

DNA or plasmid	ORF	Relative *cat* expression[a]				
		HeLa	SCC-13	18:8	LTK	CV
Carrier DNA		16	12	6	1	1
pRB3522	TIF	68	51	7	1	1
pRB420	ICP0	45	41	14	1	11
pRB3611	ICP4	21	ND[b]	4	1	1
pRB419	ICP27	17	ND	6	1	1

[a] Relative *cat* expression was normalized to pCAT3m levels. Assays were performed at 40 h posttransfection. Results represent the average fold activation of at least three separate transfections performed in duplicate. Results from individual transfections varied by less than 25%. Ten micrograms of tester plasmid was transfected with 20 μg of either carrier or HSV DNA.

[b] ND, Not determined.

TIF activation of HPV-18 is epithelial cell dependent, p18CAT1221 was also tested in SCC-13 cells, a squamous cell carcinoma cell line lacking HPV-18 sequences. When p18CAT1221 was cotransfected with carrier DNA into SCC-13 cells, a 12-fold increase in expression was observed. When cotransfected with pRB3522 (TIF) and pRB420 (ICP0), increases in expression to 51-fold and 41-fold, respectively, were observed. Activation of HPV-18 expression was similar to that observed in HeLa cells, suggesting that E6 and E7 do not contribute to HSV *trans*-activation.

The same experiments were repeated in NIH 3T3 cells and the NIH 3T3-derived cell line 18:8, which constitutively express the E6-E7 region of HPV-18. The results obtained with these two cell lines were identical and indicate that only ICP0 can activate HPV-18 expression in rodent fibroblasts (Table 1). In addition, these results confirm that the E6 and E7 gene products do not act synergistically with HSV genes to activate HPV-18 expression. To compare the levels of TIF and ICP0 gene-induced activation on HPV promoters with that seen with HSV promoters, TIF and ICP0 expression plasmids (pRB3522 and pRB420) were cotransfected with plasmids containing the ICP4 gene promoter or the *tk* promoter placed upstream of the *cat* gene (12). The TIF gene was observed to increase expression of the ICP4 gene 9-fold, while the ICP0 gene increased *tk* expression 4.5-fold.

We also examined whether other viral genes can alter the expression of p18CAT1221. Human immunodeficiency virus type 1 and human T-cell lymphotropic virus type I contain viral genes which activate the expression of their host promoters (28, 35). The human immunodeficiency virus type 1 and human T-cell lymphotropic virus type I *tat* expression plasmids (pSVETA and pHTLVtat-1, respectively) were cotransfected with p18CAT1221 into HeLa and CV-1 cells to evaluate their effects on HPV-18 expression. No activation of p18CAT1221 by pSVETA or pHTLVtat-1 was observed (data not shown).

Localization of the *cis*-acting DNA activated by the TIF and ICP0 genes. The HPV-18 sequences responsive to the TIF and ICP0 genes were localized by using a second series of constructs with heterologous promoters. When the TIF (pRB3522) or ICP0 (pRB420) gene was cotransfected with p18CATa, which contains the entire HPV-18 noncoding region placed upstream of the SV40 early promoter in the antisense orientation (Fig. 2), levels of activation similar to that seen with the homologous HPV promoter (13-fold and 16-fold, respectively) were observed. These results suggest that the HSV-responsive sequences in the noncoding region are capable of activating heterologous promoters even when placed in the antisense orientation.

To further localize the responsive sequences in the HPV-18 noncoding region, we used plasmids which contain subfragments of this region cloned upstream of pA10CAT2 (Fig. 2). Two adjoining *Rsa*I fragments of 388 and 229 bp and an *Rsa*I-*Bam*HI 242-bp fragment were cloned into the *Bgl*II site of pA10CAT2 to form p18IE6a, p18Ca, and p18RBS, respectively (Fig. 2). p18IE6a contains an element that previous studies suggest may be *trans*-activated by the HPV-18 E6 ORF, while p18Ca contains an epithelial cell-preferred enhancer element (15, 42). Plasmid p18RBS contains the proximal 242 bp of the HPV-18 E6 and E7 promoter. These three recombinant plasmids collectively contain the entire HPV-18 noncoding region (Fig. 2).

In cotransfection experiments with p18IE6a or p18RBS and the TIF gene expression plasmid (pRB3522), no change in the level of expression was observed in HeLa cells (Fig. 3). The sixfold activation of p18IE6a seen in these cells is

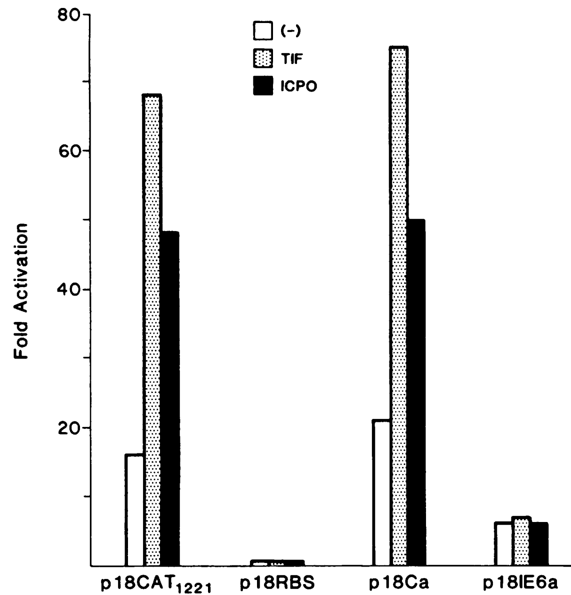

FIG. 3. Activation of HPV-18 expression by the HSV TIF and ICP0 gene products. Transfections were performed in HeLa cells, a human cell line derived from an adenocarcinoma of the cervix, with plasmid p18CAT1221, p18IE6a, p18Ca, or p18RBS. Cells were transfected with 10 μg of pA10CAT2, pSV2CAT, or the HPV-18 expression tester plasmid and either 20 μg of carrier DNA (−), HSV-1 TIF, or ICP0. The data for p18RBS, p18Ca, and p18IE6a are presented as percent acetylation normalized to that of pA10CAT2. The bar graph represents the HPV-18 expression vectors transfected with carrier, HSV-1 TIF, or HSV-1 ICP0 DNA. Results represent the average fold activation of at least three separate transfections performed in duplicate. The results of individual transfections varied by less than 25%. Assays were performed as previously described (15).

due to an E6-responsive element which was previously localized to this fragment (Fig. 1) (15). Cotransfection of p18Ca with carrier DNA resulted in a 21-fold level of activation, which increased to 75-fold with pRB3522, localizing the TIF-responsive sequences to this region (Fig. 3). When the ICP0 gene expression plasmid (pRB420) was cotransfected with p18IE6a or p18RBS, no change in expression was observed. However, when it was cotransfected with p18Ca, an increase in expression from 21-fold with carrier DNA to 50-fold with pRB420 was observed. These experiments were repeated in SCC-13, CV-1, and 18:8 cells, and similar results were observed (Table 2). Cotransfection

TABLE 2. *trans*-Activation of plasmid p18Ca by HSV-1 TIF and ICP0

DNA or plasmid	ORF	Relative *cat* expression[a]			
		HeLa	SCC-13	CV-1	18:8
Carrier DNA		21	20	1	8
pRB3522	TIF	75	50	1	ND[b]
pRB420	ICP0	50	35	10	17

[a] Relative *cat* expression was normalized to pA10CAT2 levels. Assays were performed at 40 h posttransfection. Results represent the average activation of at least three separate transfections performed in duplicate. Results from individual transfections varied by less than 25%. Ten micrograms of tester plasmid was transfected with 20 μg of either carrier or HSV DNA.

[b] ND, Not determined.

of pA10CAT2 with pRB3522 or pRB420 was observed to have no effect on the expression (data not shown). We therefore localized the TIF- and ICP0-responsive elements to the 229-bp *Rsa*I fragment contained in p18Ca.

E2 *trans*-activation in the presence of HSV gene products. The HPV-18 noncoding region contains a conditional enhancer element that is inducible in *trans* by the BPV-1 E2 ORF (15). The BPV-1 E2 gene product is a *trans*-activator equivalent to or better than the HPV-18 E2 gene product (43). To examine whether any additive interaction occurs between these viral *trans*-acting proteins, a plasmid expressing the BPV-1 E2 cDNA (pc59) was cotransfected with p18CATa and either pRB3522 or pRB420 in HeLa cells. E2 exhibits a complex pattern of positive and negative effects on the E6-E7 promoter included in p18CAT1221 (43; unpublished observations). We sought to simplify our study by concentrating on the activation effects of E2 in the enhancer tester plasmid p18CATa (Fig. 2).

Transfection of p18CATa into HeLa cells resulted in a 13-fold increase in expression above that of pA10CAT2, which increased to 64-, 43-, and 86-fold with cotransfection with pRB3522 (TIF), pRB420 (ICP0), or pc59 (E2), respectively (Fig. 4). When p18CATa was cotransfected with pc59 and pRB3522 an increase in expression to 246-fold was obtained, while with pc59 and pRB420 an increase in expression to 138-fold was observed. These results suggest a possible additive effect between the E2 gene product and the HSV TIF and ICP0 genes. However, both the TIF (6-fold; unpublished observation) and ICP0 (3- to 10-fold; 39a) genes activate the expression of pSV2CAT but not pA10CAT2 in transient assays. Therefore, since the synthesis of E2 is under control of the SV40 early promoter in pc59, we cannot exclude the possibility that the additive effect of the TIF or ICP0 gene and E2 on HPV expression is, at least in part, an indirect effect. When p18CATa was cotransfected with pRB3522 and pRB420 together, an increase in expression to 54-fold was observed (Fig. 4). This result is similar to the activation by the TIF gene alone, suggesting that no cooperative effect exists between these two HSV *trans*-acting genes and HPV-18 expression.

Activation of HPV-18 expression in the presence of TPA. Phorbol esters induce the expression of several cellular and viral genes, including c-*myc*, BPV-1, and SV40 (1, 17, 19). To determine whether TPA activates HPV-18 expression, transient assays were performed in HepG2 cells transfected with the HPV-18 promoter plasmid (p18CAT1221) and treated with TPA. HepG2 cells are very sensitive to TPA induction in transient assays (3). pSV2CAT expression increased from 86-fold without TPA to 506-fold with TPA, representing 5.9-fold by TPA induction (Fig. 5) (3, 19). Without TPA, p18CAT1221 was expressed 2.6-fold above pTKCAT, which increased to 8.0-fold with TPA, representing 3.1-fold induction by TPA.

To localize the TRE, the IE6 and C regions of the HPV-18 noncoding region were placed upstream of the heterologous pTKCAT plasmid and tested with TPA. pTKCAT was used because pA10CAT2 contains a TRE and is activated by TPA (3). Plasmids pTKIE6a and pTKCa were transfected into HepG2 cells and found to be unresponsive to TPA induction (Fig. 5). This localizes the TRE to the remaining 403-bp *Rsa*I-*Sph*I region. Examination of this region revealed a sequence which contains 7 of the 8 bp of the TRE consensus sequence located 95 bp upstream of the E6-E7 promoter TATA box (TGACTAAG) (Fig. 1) (3, 10). To examine whether this sequence is responsive to TPA, a 30-bp oligonucleotide containing the HPV-18 TRE was cloned into the

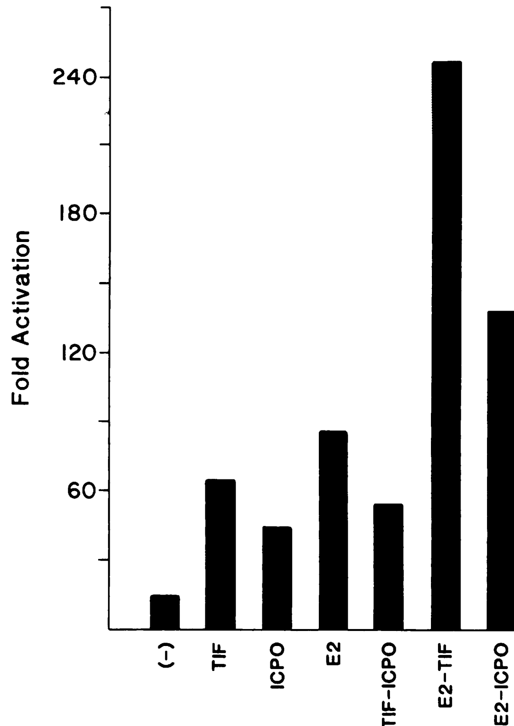

FIG. 4. Effect of E2 and TIF or ICP0 on activation of HPV-18. Transfections were performed in HeLa cells with p18CATa, which contains the HPV-18 noncoding region within a 1.1-kilobase *Bam*HI fragment placed in the *Bgl*II site of pA10CAT2 in the antisense orientation. Cells were transfected with (i) 10 μg of p18CATa and carrier DNA (20 μg) or carrier DNA (10 μg) with TIF, ICP0, or E2 (10 μg) or (ii) E2 (10 μg) with plasmids expressing either TIF or ICP0 (10 μg).

*Bam*HI site of pTKCAT to create pTK-TPA (Fig. 5). When pTK-TPA was transfected into HepG2 cells, an increase in expression of 3.4-fold above that seen with pTKCAT was observed, which in the presence of TPA increased to 14.4-fold, demonstrating 4.2-fold TPA induction (Fig. 5). We have designated this element IETPA, for a TPA-inducible element. We cannot exclude the possibility that additional TREs which lack sequence homology to previously identified TREs are present in the 403-bp *Rsa*I-*Sph*I fragment.

TPA-induced effects on cells that stably express HPV-18. While NIH 3T3 cells that express p18PEpolyA (18:8 cells, e.g.) grow in soft agar and form tumors in nude mice, they exhibit minimal morphological transformation (6) and are unable to form foci in tissue culture. In contrast, when the corresponding region of HPV-16 is expressed from a strong heterologous promoter, morphological transformation is observed (26). We sought to investigate whether a TPA-induced increase in HPV-18 expression could produce similar results in 18:8 cells. TPA (240 ng/ml) was added to NIH 3T3 and 18:8 cells, and the cells were monitored for 5 days. The cells were maintained in 10% FCS, in contrast to the 0.5% FCS used in TPA *trans*-activation experiments, since 18:8 cells cultured in low serum with TPA become rounded and detach after 36 h and NIH 3T3 cells grow poorly in low serum. NIH 3T3 cells with or without TPA displayed similar morphologies and did not form foci (Fig. 6). When TPA was added to 18:8 cells, they appeared to be very refractile and foci were apparent after 2 days, while at lower concentrations foci appeared after 4 days (Fig. 6).

To determine whether TPA increased expression of the HPV-18 E6-E7 region, 18:8 cells were examined by Northern analysis with or without TPA. Cells were incubated at a concentration of 240 μg of TPA per ml and were harvested at 16 h. A roughly fourfold increase in E6-E7 expression was

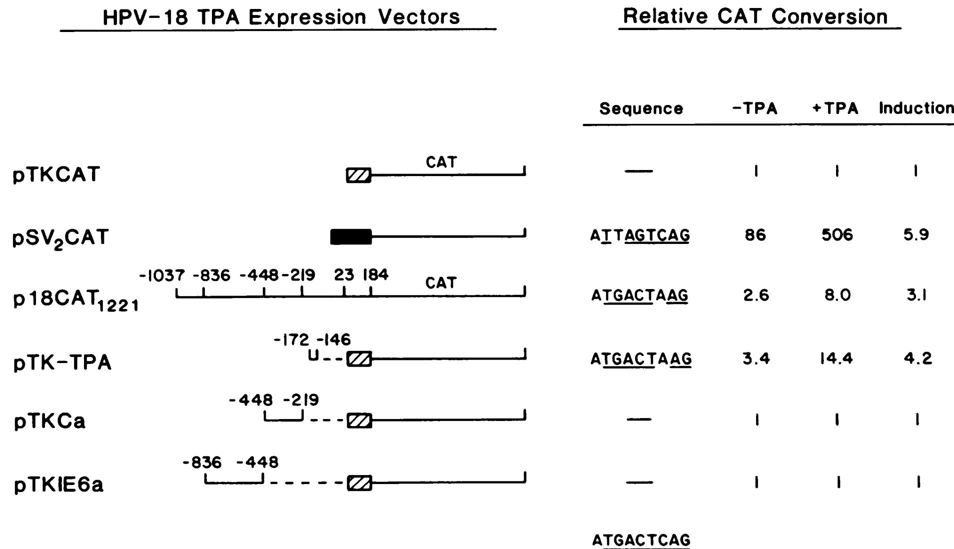

FIG. 5. Constructs used to test for TPA induction of HPV-18 and relative amount of TPA induction. Plasmid pTKCAT (3) contains the HSV thymidine kinase gene promoter placed upstream of *cat*, while pSV2CAT (15) contains the SV40 promoter and the 72-bp tandem repeats placed upstream of *cat*. p18CAT1221 contains a 1,221-bp *Bam*HI-*Sph*I fragment from +184 to −1,037 bp upstream of the E6-E7 transcription start site cloned upstream of *cat*. pTK-TPA contains a 30-bp synthetic oligomer containing the HPV-18 TRE placed into the *Bam*HI site of pTKCAT. pTKIE6a and pTKCa contain the 388- and 229-bp *Rsa*I fragments placed into the *Bam*HI site of pTKCAT. The underlined sequences represent the promoter sequences of SV40 and HPV-18 that are homologous to the 8-bp consensus TRE sequence from the human metallothionein IIa promoter (3). Cells were transfected with 4 μg of tester DNA, and assays were performed as previously described. Relative *cat* expression was normalized to the level of pTKCAT. Results represent the average fold activation of at least three separate transfections performed in duplicate. Results from individual transfections varied by less than 25%.

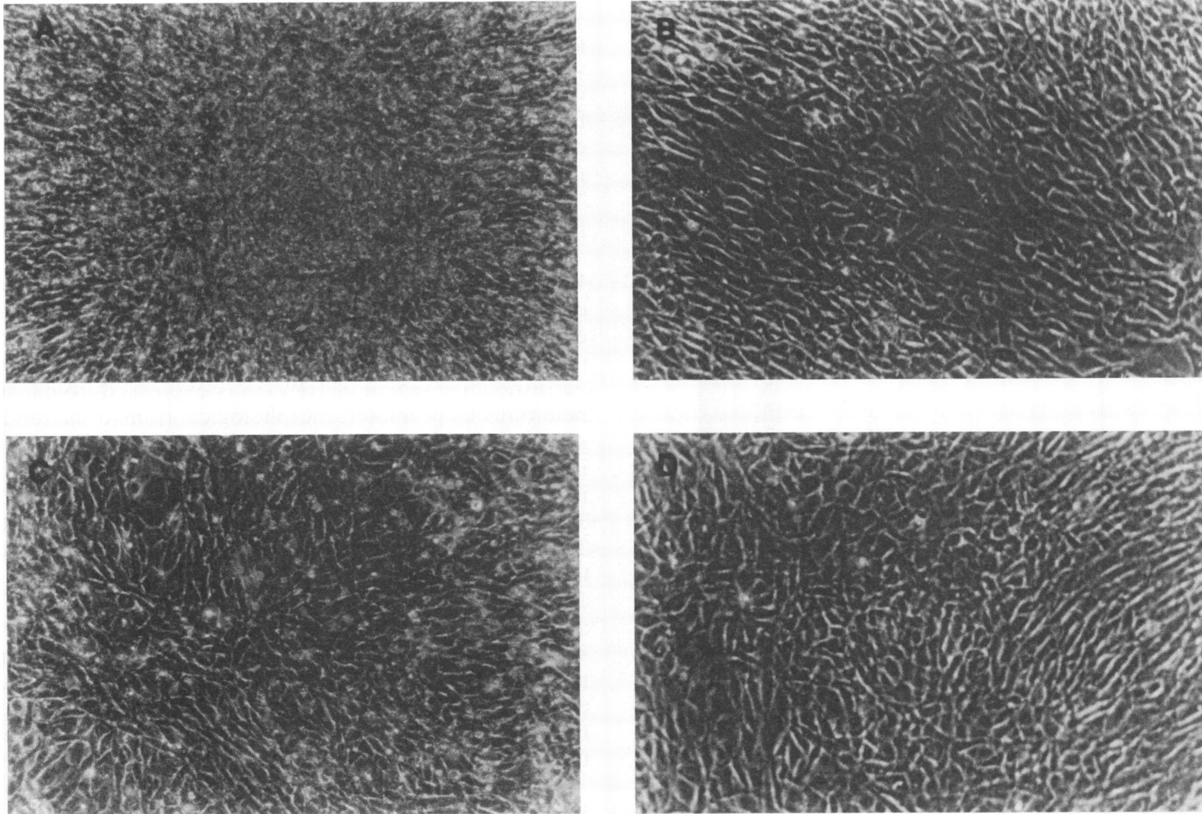

FIG. 6. Morphology of NIH 3T3 cells (B and D) and 18:8 cells, which express the HPV-18 E6-E7 region (A and C), after 5 days of induction with 240 ng of TPA per ml (A and B) and without TPA (C and D). Magnification, ×135.

observed in 18:8 cells incubated with TPA (Fig. 7). These results suggest that TPA increases the expression of stably integrated copies of HPV-18, resulting in increased morphological transformation of 18:8 cells.

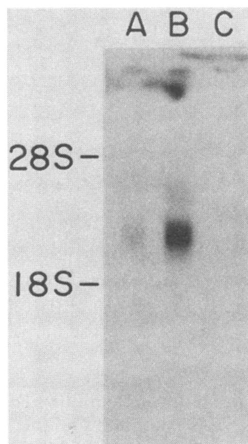

FIG. 7. Northern analysis of HPV-18 expression in 18:8 cells incubated without (lane A) or with (lane B) 240 μg of TPA per ml for 16 h and NIH 3T3 cells (lane C). A 20-μg sample of total cellular RNA was run on a formaldehyde-agarose gel, blotted to nitrocellulose, and probed with a 1,578-bp *Hpa*II fragment from the HPV-18 E6-E7 region. The positions of 18S and 28S rRNAs are indicated.

DISCUSSION

HPV infection alone is probably not sufficient for induction of cervical carcinogenesis, and a second event may be required. As an example, epidemiological studies have shown that HPV-16- or HPV-18-infected women who smoke have a 5- to 10-fold-higher rate of cervical cancer than do infected women who do not smoke (45). The possible role of a second event has also been shown experimentally in HPV-16- and HPV-18-mediated transformation of primary rodent cells. While HPV-16 and HPV-18 sequences are capable of immortalizing rodent cells, they were unable to fully transform these cells (6, 23, 26). Instead, HPV-16 and HPV-18 require the presence of a cooperating oncogene, such as *ras*, for complete cellular transformation (26; M. Bedell, personal communication). In the present study, we examined whether other viral or chemical factors can activate HPV-18 gene expression and may act as cooperating agents in transformation.

The ability of one virus to augment the expression of another virus or rescue it from a latent state has previously been shown. Adenovirus and HSV can activate the expression of the human immunodeficiency virus type 1 and human T-cell lymphotropic virus type I long terminal repeats, respectively (9, 31). While HSV infection of genital epithelia probably does not result in malignancy, studies suggest that it may act as a cofactor. This is supported by the presence of both HSV and HPV DNA sequences in a small percentage of cervical tumor cells (25, 34). Cervical cells are permissive to both HSV and papillomavirus infections, and the possibility

exists that both viruses can be transcriptionally active in the same cell (21, 34, 48). HSV gene products have been shown to code for *trans*-acting factors which activate the immediate-early genes of HSV (5) and non-HSV promoters (14, 32) and include TIF, a structural component of the virion, ICP0, ICP4, and ICP27 (22, 31). In this study, we concentrated on HSV-1 viral *trans*-activators, but we suspect that similar results would be observed with HSV-2. This similarity is supported by studies indicating that ICP0s from HSV-1 and HSV-2 are functionally interchangeable during productive viral infection and act by identical mechanisms (40).

In our studies, ICP0 and TIF were found to activate HPV-18 expression. Activation by TIF appeared to be cell type specific and limited to epithelial cell lines, HeLa and SCC-13. The responsive elements were localized to a 229-bp *Rsa*I fragment of the HPV-18 noncoding region, which also contains an epithelial cell-preferred enhancer (15, 42). Examination of this fragment revealed an 18-bp region (GCTTGCaTAACTATATCC) with a high degree of homology to the HSV-1 consensus sequence (TIC; *trans*-induction *cis* site) that is activated by TIF (Fig. 1) (22). Small variations on the TIF consensus sequence have been found in nonherpesvirus genes which are responsive to TIF (32). The cell type specificity displayed by TIF is surprising, since TIF activates HSV promoters in a variety of cell types, including fibroblasts (22). This suggests that for HPV-18 *trans*-activation, TIF may interact with transcriptional regulatory proteins that are present only in epithelial cells. Activation of HPV expression by TIF and ICP0 was observed when the HPV-responsive elements were located upstream of their homologous promoter as well as when they were placed in the antisense orientation upstream of the heterologous SV40 early promoter. The ability of TIF and ICP0 to activate expression in these latter constructs suggests that these elements act in a manner similar to that of other mobile inducible elements.

Activation of HPV-18 expression by ICP0 was observed in epithelial (HeLa and SCC-13) and nonepithelial (CV-1 and NIH 3T3) cells. Interestingly, the region of HPV-18 activated by ICP0 is the same 229-bp fragment activated by TIF. No *cis*-acting DNA sequence has been identified for ICP0, and previous studies suggested that ICP0 acted as a nonspecific *trans*-acting gene (32). Since TIF and ICP0 activate HPV-18 expression in different cell types, their mechanisms of *trans*-activation are probably different. When TIF and ICP0 were cotransfected simultaneously with p18CATa, no additive effect on expression was observed. This suggests that either one HSV-encoded factor has priority over the other or while TIF and ICP0 may function by different mechanisms, they may act upon the same saturated site. We are attempting to separate the HSV TIF- and ICP0-responsive sequences from one another to determine whether they are distinct elements. In contrast, when either TIF or ICP0 was cotransfected with E2, there appeared to be an additive effect on HPV-18 expression.

Phorbol esters act to potentiate the effects of carcinogens at subtumorigenic concentrations (46). TPA activates the transcription of several viral and cellular genes by a mechanism involving the activation of protein kinase C (3, 7, 19, 29, 49). When TPA was added to HepG2 cells transfected with p18CAT1221, an increase in expression of 3.1-fold was observed above that of cells transfected in the absence of TPA. TPA induction experiments were performed in HepG2 cells because they are extremely sensitive to TPA *trans*-activation in transient assays (19, 20). The HPV-18 responsive element was localized to a 30-bp region (designated IETPA) which contains a sequence similar to previously identified TPA-inducible elements (3, 24). The level of TPA induction of HPV-18 is slightly less than that observed with other promoters that contain TRE sequences and may be due to a 1-bp mismatch at position six of the consensus TRE (3).

Addition of TPA to NIH 3T3 cells expressing the HPV-18 E6-E7 region from their homologous promoter (18:8 cells) resulted in an increase in steady-state RNA levels and a change in their cellular appearance. When TPA was added to 18:8 cells they appeared more refractile and formed foci after 2 days, while at lower concentrations foci appeared after 4 days. Untreated 18:8 cells and TPA-treated NIH 3T3 cells appeared to be similar to NIH 3T3 cells. Similar changes in morphology were observed when HPV-16 genes were overexpressed from a strong long terminal repeat promoter (26). We cannot, however, exclude the possibility that TPA also activates additional cellular genes that may contribute to this altered phenotype (46). In preliminary experiments, cotransfection of ICP0 and p18PEpolyA into NIH 3T3 cells resulted in a high frequency of focus formation, suggesting that different viral genomes also cooperate in the induction of morphological transformation (Gius, unpublished data). Several forms of chemical and viral cofactors may thus influence the HPV-induced transformation state by altering papillomavirus expression.

Malignant transformation is a multistep process which may result from a wide variety of cellular abnormalities, carcinogenic agents, or viral infections. Each is (or perhaps many are) required to induce a normal cell to become neoplastic. The events that trigger the transition from a benign to a malignant HPV-induced lesion remain poorly defined and may involve various forms of transcriptional activation. An increase in HPV-18 gene expression may be the second event necessary to induce a morphological change from a normal cell type to a transformed phenotype. In this study, we demonstrated that two HSV genes and a phorbol ester can stimulate HPV-18 expression and thus may play a role in the development of HPV-18-induced malignancies.

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