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Transcriptional *trans*-Activation by the Human Papillomavirus Type 16 E2 Gene Product

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We identified a conditional transcriptional enhancer in the long control region (LCR) of human papillomavirus type 16 (HPV-16). This conditional enhancer requires activation in *trans* by a product of the viral early-region open reading frames (ORFs). Primer extension analysis of chloramphenicol acetyltransferase RNA isolated from transiently transfected CV-1 cells demonstrated that *trans*-activation of the HPV-16 LCR enhancer operated at the transcriptional level. Mutational analysis of the early ORFs demonstrated that the conditional enhancer of the LCR was *trans*-activated by the product of the E2 ORF. The E2 gene product of bovine papillomavirus type 1, which can *trans*-activate the conditional enhancer in the bovine papillomavirus type 1 LCR, was also capable of *trans*-activating the E2-responsive enhancer of HPV-16. The activity of the HPV-16 LCR enhancer was also assayed in two human cervical carcinoma cell lines, HeLa and SiHa, which harbor transcriptionally active, integrated HPV-18 and HPV-16 DNA sequences, respectively. No endogenous E2 or E2-like activity was detected in either cell line.

The papillomaviruses are a group of small DNA tumor viruses which induce benign epithelial tumors or warts in a wide variety of vertebrate hosts, including humans (for a review, see reference 26 or 34). A subgroup of the papillomaviruses are associated with benign lesions which under certain circumstances may progress to malignant carcinomas. This subgroup includes the Shope rabbit papillomavirus, bovine papillomavirus type 4 (BPV-4), and certain human papillomavirus (HPV) types.

Recently, evidence has accumulated which strongly implicates several HPV types in malignant as well as benign human genital tract lesions. HPV type 6 (HPV-6) and HPV-11 have generally been detected in mild cervical dysplasias and condyloma acuminata, lesions which appear rarely to progress to malignancy (16). HPV-31 DNA has very recently been found in several cases of mild and moderate dysplasia and in 6% of invasive cervical cancers (28). DNA of HPV types 16, 18, and 33 has been identified in most cases of severe dysplasia and carcinoma in situ of the cervix and in greater than 70% of the cervical carcinomas examined as well as in some vulvar and penile carcinomas (4, 5, 12). HPV-16 and HPV-18 DNA sequences have also been detected and analyzed in several cell lines established from human cervical carcinomas (3, 5, 33, 37, 38, 51).

Detailed molecular studies of the biology of the papillomaviruses have been hindered owing to the lack of a permissive tissue culture system which can support the full viral replication cycle. The papillomaviruses are highly epitheliotropic; the expression of the productive, late viral functions appears to depend on factors restricted to fully differentiated squamous epithelial cells. The induction of cellular proliferation in virus-infected benign and virus-associated malignant lesions may be due to subversion of the normal cellular control of epithelial differentiation, presumably mediated by one or more of the early gene products of the virus.

One notable feature of the papillomavirus-host cell interaction is the stable maintenance of the viral genome in an extrachromosomal state in transformed cells (27). In benign as well as in malignant tumors of Shope rabbit papillomavirus-infected rabbits and of patients with epidermodysplasia verruciformis infected with HPV-5, the viral genomes exist as free monomers or oligomers (32, 46, 48). In contrast, in cervical carcinoma tissues and in cell lines derived from cervical carcinomas, the HPV-16, -18, and -33 viral DNA is integrated into the host cell genome (4, 5, 30, 37, 38, 51). In these carcinomas and derived cell lines, integration of the HPV genome has commonly occurred within the E1 or E2 open reading frames (ORFs) of the viral genome, with the resulting disruption or substantial deletion of the E2 ORF (3, 38).

The E2 ORF is well conserved in each of the papillomavirus genomes sequenced thus far. The E2 gene of BPV-1 has been shown to encode a transcriptional *trans*-activation function which can stimulate a viral conditional enhancer present in the long control region (LCR) located 5′ to the early ORFs (43). To determine whether this transcriptional regulatory system is a unique characteristic of BPV-1 or a general feature shared by the papillomaviruses, we undertook an analysis of the corresponding E2 and LCR regions of the HPV-16 genome. In this study, we identified a conditional transcriptional enhancer in the HPV-16 LCR which is responsive to the HPV-16 E2 gene product. Furthermore, we demonstrated that the BPV-1 E2 gene product was capable of *trans*-activating the conditional enhancer of HPV-16, suggesting that both the E2 gene product and its putative target site in the LCR are well conserved. Finally, we examined whether human cervical carcinoma cell lines harboring transcriptionally active HPV sequences contain factors which can activate the HPV-16 E2-responsive LCR enhancer in the absence of added exogenously expressed E2.

MATERIALS AND METHODS

Cell culture. African green monkey kidney CV-1 cells were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) with 10% fetal bovine serum and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The human cervical carcinoma cell lines SiHa and HeLa were obtained from the American Type Culture

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Collection and grown in the same medium as previously described (51).

Construction of CAT recombinant plasmids. The $pA_{10}CAT$ plasmid containing the chloramphenicol acetyltransferase (CAT) gene positioned behind the simian virus 40 (SV40) enhancer-deleted early promoter has been previously described (25). The plasmid pSV2CAT containing the complete SV40 early promoter with the enhancer positioned 5′ to the CAT gene has also been previously described (18). A DNA fragment (nucleotides [nts] 7007 to 57) corresponding to the LCR of HPV-16 from the clone described by Durst et al. (12) was cloned into $pA_{10}CAT$ at the *Bgl*II (5′) or *Bam*HI (3′) site in each orientation, generating the plasmids p863.1, p864.1, p865.1, and p865.2 (Fig. 1). Plasmids p858 and p859 containing the HPV-16 early ORFs downstream of the SV40 early promoter were constructed by using the *Taq*I-to-*Stu*I fragment and the *Tth*III-to-*Stu*I fragment as depicted in Fig. 1. Mutations were created in p858 and p859 by restriction site fill-in repair (p1026), restriction fragment deletion (p1058), or insertion of a translational termination linker (TTL), 5′ TTAGTTAACTAA 3′, (p1013, p1016, p1027, and p1028). The BPV-1 plasmids p407.1 and C59 and the C59 mutants (C59-2878 and C59-3881) have been described previously (43, 49, 50). Bacterial transformation of *Escherichia coli* K-12 HB101 (20), recombinant screening, propagation of recombinant plasmids, and nucleic acid manipulations were performed by conventional methods (29).

Mammalian cell transfections. DNA transfections were performed by calcium phosphate coprecipitation (19) with a total of 10 μg of DNA per 60-mm dish (5 μg of CAT plasmid plus 5 μg of the *trans*-activation plasmid or pBR322). At 4 h after transfection, the cells were treated with 15% glycerol (13) for 1 min. After the monolayer was washed twice with fresh medium, the cells were incubated in complete medium supplemented with 5 mM sodium butyrate (pH 7.0) for 48 h (17).

CAT assays. CAT assays were performed as previously described (18, 25). Briefly, cell extracts were incubated with [^{14}C]chloramphenicol (50 mCi/mmol; Amersham Corp.) and 4 mM acetyl coenzyme A (Pharmacia, Inc.) in 250 mM Tris hydrochloride (pH 7.8) at 37°C for up to 60 min. The products of the acetylation reaction were separated by ascending thin-layer chromatography, and the fractionated products were localized by autoradiography. Acetylated and unacetylated spots were excised for quantitation by liquid scintillation. Kinetic analyses were performed by using a uniform quantity of cellular extract (15 μg of total protein) measured by Bio-Rad protein determination (Bio-Rad Laboratories). The extracts were incubated with [^{14}C]chloramphenicol in a standard reaction and sampled at various times, and the percent acetylation was determined. For each experiment, pSV2CAT and $pA_{10}CAT$ were used as positive and negative controls, respectively.

RNA extraction. Total RNA was extracted from cells 48 h after transfection by lysis in 4 M guanidine isothiocyanate–0.5% sodium *N*-lauroylsarcosine–25 mM sodium citrate (pH 7.0)–0.1 M 2-mercaptoethanol. High-molecular-weight DNA was sheared by several passages through an 18-gauge needle to reduce viscosity. The lysate was layered over a cushion of 5.7 M CsCl–100 mM EDTA (pH 7.0) in an SW41 polyallomar tube. RNA was pelleted by centrifugation at 30,000 rpm for 20 h at 17°C in a Spinco ultracentrifuge (7). The RNA pellets were suspended in distilled water and precipitated at −20°C by the addition of a 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol.

Primer extension analysis. A 20-base oligonucleotide complementary to a portion of the 5′ end of the CAT gene was synthesized (5′ TCCATTTTAGCTTCCTTAGC 3′). The oligonucleotide primer was 5′ end-labeled with polynucleotide kinase (P-L Biochemicals, Inc.) under the following conditions: 50 mM Tris hydrochloride (pH 9.0), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 0.08 μg of the synthesized oligonucleotide, and 5 U of kinase. The incubation was carried out at 37°C for 1 h, and the reaction was stopped by heating at 70°C for 10 min. The 5′-end-labeled primer (0.004 μg) was annealed to the RNA by mixing in 100 mM NaCl, 20 mM Tris hydrochloride, 0.1 mM EDTA and heating to 90°C for 3 min. This mixture was then incubated at 55°C for 10 min and cooled slowly to room temperature (approximately 40 min). The annealed RNA and primer mixture was then placed directly into a primer extension reaction consisting of 20 μg of total RNA, 0.004 μg of oligonucleotide primer, 50 mM Tris hydrochloride (pH 7.5), 100 μg of bovine serum albumin per ml, 3 mM $MgCl_2$, 75 mM KCl, 50 μg of actinomycin D per ml, 0.5 mM deoxynucleotide triphosphates, 10 mM dithiothreitol, and 4,000 U of reverse transcriptase per ml (cloned murine leukemia virus; Bethesda Research Laboratories, Inc.) in a 50-μl volume. Incubation was at 37°C for 1 h. The reaction was stopped by the addition of EDTA to 25 mM; it was extracted with phenol-chloroform and ethanol precipitated. The primer extension products were fractionated through an 8% polyacrylamide-8 M urea gel.

RESULTS

LCR of HPV-16 contains a conditional enhancer element. To analyze the potential transcriptional enhancer elements contained within the LCR of HPV-16, a fragment corresponding to the entire LCR region from the *Pst*I site (nt 7007) to the *Hpa*II site (nt 57) was cloned into the plasmid $pA_{10}CAT$, which contains the SV40 enhancer-deleted SV40 early promoter (Fig. 1) in front of the bacterial CAT gene. This LCR fragment was cloned in either the transcriptional sense or antisense orientation at the 5′ *Bgl*II site or the 3′ *Bam*HI site relative to the CAT gene. The four resulting HPV-16 LCR/CAT plasmids (p863.1, p864.1, p865.1, and p865.2) are depicted in Fig. 1. In addition, since the analogous enhancer element in the BPV-1 LCR is conditional and responsive to the viral E2 gene product, segments of the HPV-16 early region containing the intact E2 ORF were cloned downstream of the SV40 early promoter in plasmid p770.1 (Fig. 1). The putative *trans*-activator plasmid p858 contained the intact E2 ORF as well as the overlapping E4 and downstream E5 ORFs and the upstream E7 and E1 ORF regions. Another putative *trans*-activator plasmid, p859, contains a segment of HPV-16 from nt 2711, 14 base pairs upstream of the beginning of the E2 ORF, and therefore should be capable of expressing the intact E2 ORF. It also contains the intact E4 and E5 ORFs.

Each of the HPV-16 LCR/CAT plasmids was transfected into CV-1 cells and assayed for CAT activity. No significant levels of CAT activity above that obtained with $pA_{10}CAT$ were detected in cells transfected with any of these four HPV-16 LCR/CAT plasmids alone (Table 1). However, in the presence of either p858 or p859 there was significant activation of each of the HPV-16 LCR/CAT plasmids, indicating that the HPV-16 LCR contained an element which could activate a heterologous SV40 early promoter in an orientation- and position-independent manner, but only in the presence of a plasmid expressing HPV-16 early gene products.

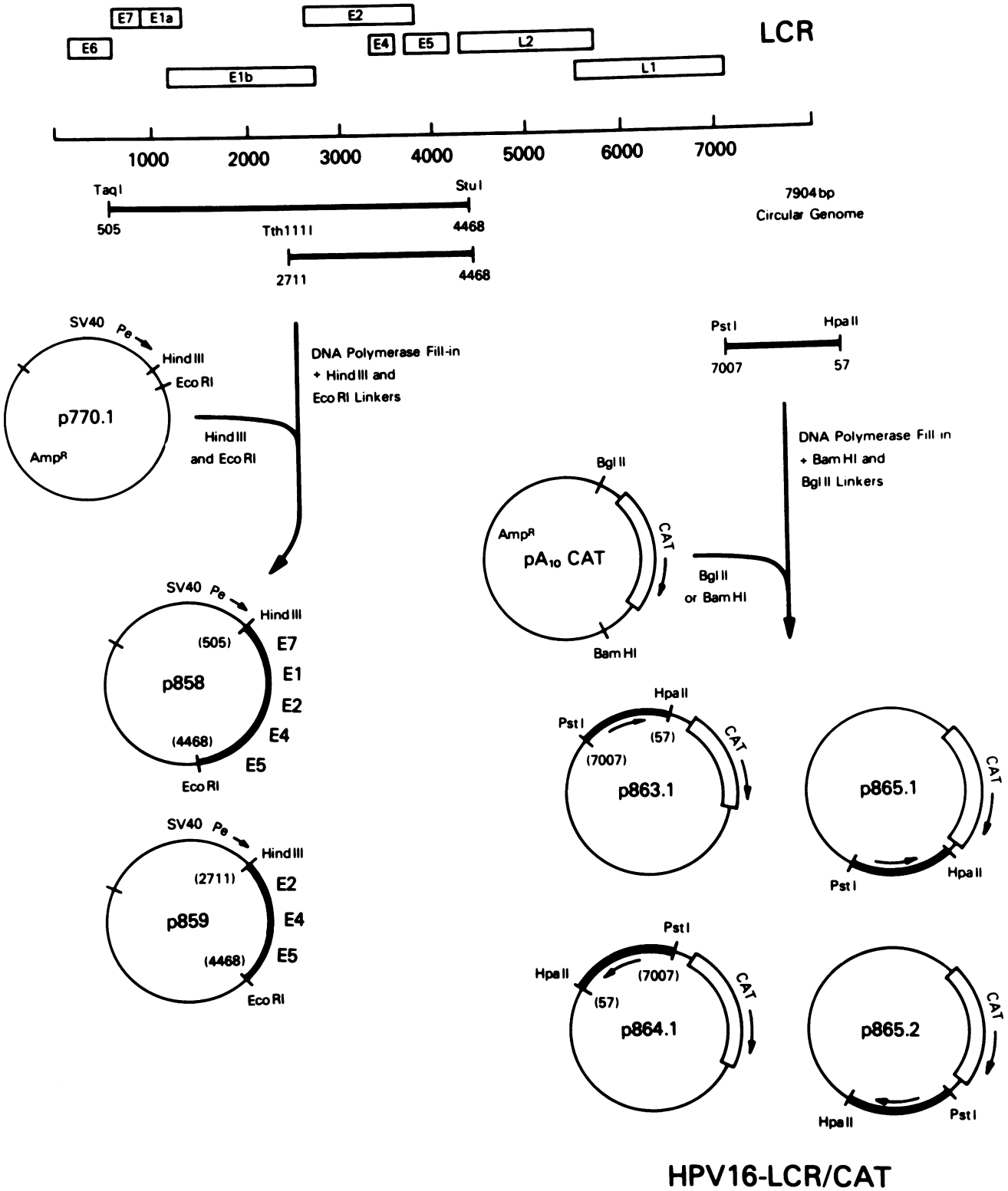

FIG. 1. Schematic diagram of plasmid constructions. The genomic map at the top of the figure is derived from the DNA sequence data of Seedorf et al. (39). The early (E1 to E7) and late (L1 and L2) ORFs are indicated; in this isolate the E1 ORF is split into the E1a and E1b ORFs (39) and differs from other HPV-16 cloned isolates, which contain an intact E1 ORF (3, 30). The LCR (nts 7152 to 65) which was previously called the noncoding region and which has also been referred to as an upstream regulatory region is also indicated. On the right, the construction of the HPV-16 LCR/CAT plasmids is depicted. The restriction fragment *Pst*I (nt 7007) to *Hpa*II (nt 57) was blunt-ended by using T4 DNA polymerase; *Bam*HI and *Bgl*II linkers were sequentially added at the *Pst*I and *Hpa*II sites, respectively. The resulting HPV-16 LCR *Bam*HI-to-*Bgl*II fragment was then cloned into the enhancer-deleted plasmid pA_{10}CAT in both the sense and antisense orientations at the 5′ *Bgl*II site and at the 3′ *Bam*HI site as indicated. The transcriptional orientations of the LCR and the CAT gene are indicated by arrows.

TABLE 1. HPV-16 *trans*-activation

Plasmid	% Conversion with:		
	Salmon sperm DNA (5 μg)	p858	p859
$pA_{10}CAT$	1.1	ND[a]	ND
pSV2CAT	97.0	ND	ND
HPV-16 LCR/CAT			
p863.1 (5'S)[b]	<2	54.4	24.8
p864.1 (5'AS)[b]	<2	20.3	19.0
p865.1 (3'AS)	<2	10.8	9.2
p865.2 (3'S)	<2	27.4	10.0

[a] ND, Not determined. In a separate set of experiments, $pA_{10}CAT$ was cotransfected with pBR322, p770.1 (containing the SV40 control region), and p858. The levels of CAT conversion were equivalent, indicating that p858 does not activate the enhancer-deleted SV40 early promoter.

[b] S, Sense orientation; AS, antisense orientation.

Quantitative analysis of CAT RNA. To evaluate the mechanism of *trans*-activation of the HPV-16 LCR, the steady-state levels of CAT RNA promoted from the SV40 early promoter were examined in the transfected cells. Quantitative primer extension analyses were performed by using a synthesized 20-base oligonucleotide complementary to a region in the 5' end of the CAT gene. The results of such an experiment are shown in Fig. 2. RNAs generated from the normal SV40 early start sites (15) in the HPV-16 LCR/CAT plasmids would serve as templates for the production of two primer extension products 95 and 100 nts long. Such products were evident in the analysis of RNA from the control pSV2CAT-transfected CV-1 cells (Fig. 2, lane 2). These RNA species were not detectable in CV-1 cells transfected either with $pA_{10}CAT$ alone (lane 1) or with the HPV-16 LCR/CAT plasmid in the absence of p858 or p859 (lane 3). Analysis of the RNA from CV-1 cells cotransfected with the HPV-16 LCR/CAT plasmid p863.1 in the presence of p858 (lane 4), however, demonstrated these same two primer extension products, indicating that the HPV-16 LCR enhancer stimulated production of CAT-specific mRNA from the normal SV40 early start sites. The larger primer extension product (approximately 120 nts long) was observed in all reactions, including mock-transfected CV-1 cells, indicating that the sythesis of this product was due to annealing of the primer to a cellular RNA and was not a measure of the levels of CAT RNA. The primer extension analyses are quantitative since the oligo primer is in large molar excess. Furthermore, the quantity of specific primer products obtained from these reactions was shown to be directly proportional to the amount of specific RNA in a reconstruction experiment by using increasing amounts of cellular RNA containing the CAT transcript (data not shown). Therefore, the *trans*-activation of the HPV-16 LCR enhancer by p858 was mediated by an increase in the steady-state level of CAT-specific mRNA.

Genetic mapping of the *trans*-activation function to the E2 ORF. Precise localization of the *trans*-activating function

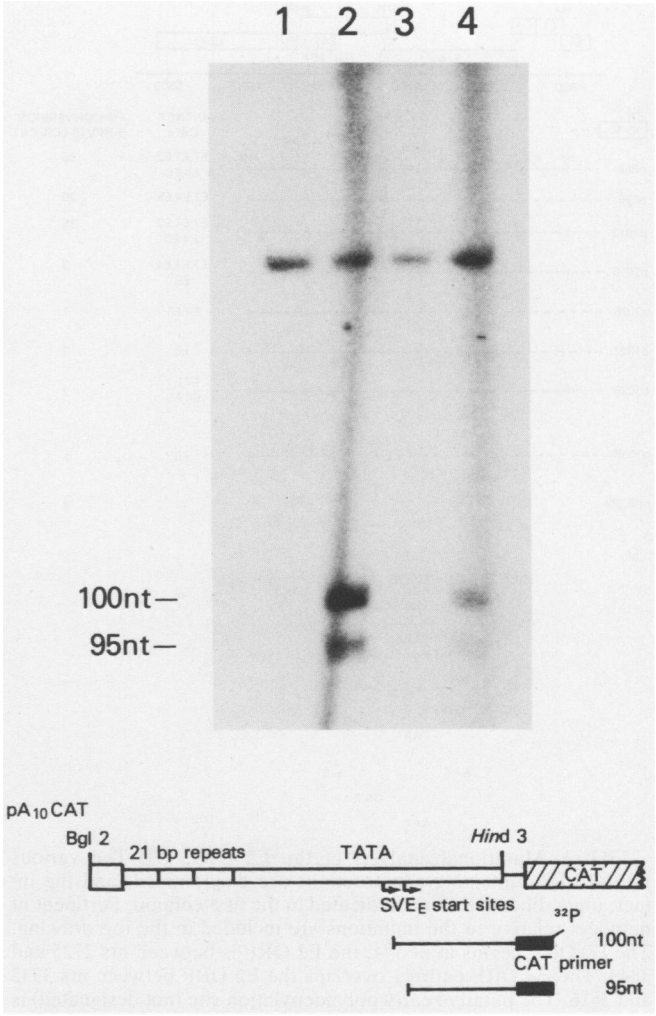

FIG. 2. Primer extension analysis of CAT RNA. CV-1 cell cultures (150 mm^2) were transfected by the standard calcium phosphate technique. The cells were glycerol shocked and incubated with 5 mM sodium butyrate as described in Materials and Methods. At 48 h after DNA addition, the cells were washed once with phosphate-buffered saline and lysed in guanidine thiocyanate solution. RNA was purified by ultracentrifugation, ethanol precipitated at least twice, and stored at −70°C in distilled water. Up to 20 μg of total RNA was used in each primer extension reaction. Labeled primer extension products were separated in an 8% denaturing polyacrylamide gel, and autoradiography was for 1 to 7 days without an intensifying screen. The sizes of the expected primer extension products are indicated by arrows and correspond to those depicted in the schematic drawing of the $pA_{10}CAT$ plasmid below. Size markers consisted of DNA sequencing ladders derived from unrelated DNAs. Lanes: 1, $pA_{10}CAT$; 2, pSV2CAT; 3, p863.1 plus pBR322; 4, p863.1 plus p858.

On the left, the construction of the *trans*-activator expression plasmids is shown. Two fragments of the HPV-16 early region consisting of the *Taq*I (nt 505) to *Stu*I (nt 4468) and the *Tth*III I (nt 2711) to *Stu*I (nt 4468) fragments were cloned downstream of the SV40 early promoter contained in the plasmid p770.1. The vector p770.1 includes the SV40 control region from nt 5171 (*Hin*dIII) to nt 346 (*Hpa*II) cloned in a pML2 background. The vector also contains the SV40 late polyadenylation site (nts 3533 to 2668) on the SV40 late promoter side to facilitate utilization of the late promoter for expression (not applicable to this study). These sites within the HPV-16 genome were blunted with T4 DNA polymerase, and *Hin*dIII and *Eco*RI linkers were added sequentially. The direction of RNA transcription from the SV40 early promoter (Pe) is designated. The plasmid p858 contains intact E7, E1a, E1b, E2, E4, and E5 ORFs, while the shorter plasmid p859 contains only E2, E4, and E5 ORFs. In addition, each of these HPV-16 early-region fragments contains the putative early polyadenylation recognition site (AATAAA) located at nt 4213.

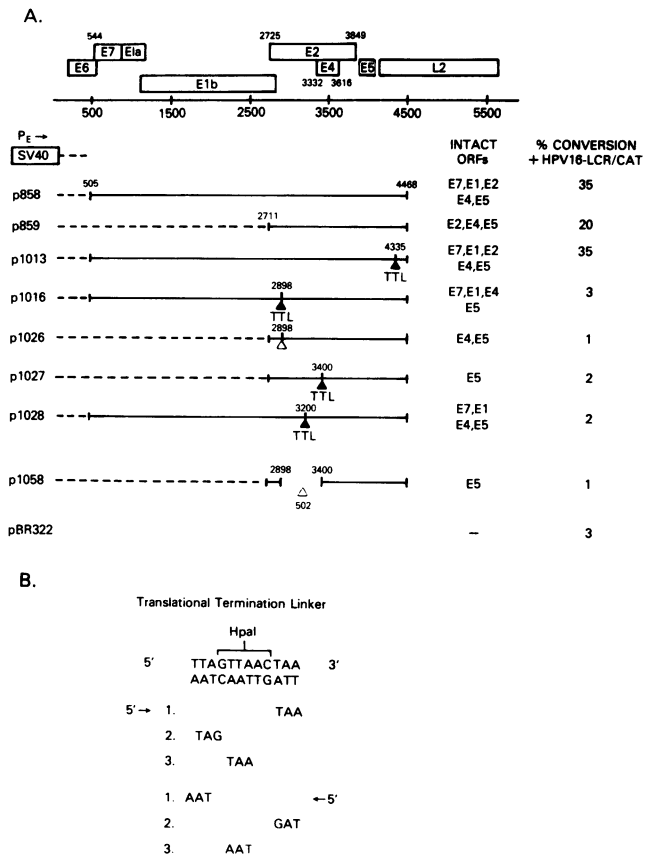

FIG. 3. Mutational analysis of the E2 ORF. (A) The various HPV-16 E2-containing constructions are diagrammed, and the intact, unmodified ORFs are indicated in the first column. Pertinent nt numbers relative to the mutations are included in the top drawing. The E7 ORF begins at nt 544; the E2 ORF is between nts 2725 and 3849. The E4 ORF entirely overlaps the E2 ORF between nts 3332 and 3616. The putative early polyadenylation site (not designated) is at nt 4213. Cotransfection experiments with the designated mutant DNAs in the presence of p863.1, the HPV-16 LCR/CAT, were conducted, and the percent acetylation from a typical experiment is shown in the column at the right. TTL, Insertion of a TTL; △, nt deletion. All constructions were verified by detailed restriction enzyme analysis and DNA sequencing. (B) The nt sequence of the TTL is shown. The oligonucleotide is self-complementary, requiring that only one strand be synthesized. The TTL contains an internal *Hpa*I site to facilitate recombinant screening. As shown, termination codons are found in all three reading frames regardless of orientation.

within the HPV-16 early ORFs was accomplished by mutational analyses (Fig. 3). A frameshift mutation was created in the 5′ portion of the E2 ORF at the *Bst*XI site (nt 2898) of p859 to generate p1026. A 502-base-pair deletion mutation affecting the E2 and E4 ORFs was generated in p859 by deleting the *Bsx*XI-to-*Bal*I fragment (nts 2898 to 3400) to generate p1058. A TTL was inserted into p858 at the *Bst*XI site (nt 2898), the *Hin*cII site (nt 3200), and the *Mst*II site (nt 4335) to create the plasmids p1016, p1028, and p1013, respectively. Insertion of the TTL at the *Bal*I site (nt 3400) of p859 generated the plasmid p1027 (Fig. 3). The nt sequence of the TTL is shown in Fig. 3B. This linker is similar in design to one previously described by this laboratory (36).

Each of these plasmids was transfected into CV-1 cells with the HPV-16 LCR/CAT plasmid p863.1 and assayed for its ability to activate CAT expression (Fig. 3A). The highest levels of *trans*-activation were obtained with p863.1 cotransfected with p858, leading to an induction of CAT 10- to 15-fold over the background obtained with pBR322. The shorter expression plasmid p859 stimulated CAT expression 5- to 6-fold above background levels, somewhat lower than

FIG. 4. *trans*-activation of the HPV-16 LCR by the BPV-1 E2 gene product. Two HPV-16 LCR/CAT constructions were used, i.e., p863.1, described in the legend to Fig. 1, and p1015, derived from p863.1 and containing only the right half of the LCR (nts 7465 to 57). The BPV-1 cDNA clone C59 has been previously described (49) and includes an unspliced BPV-1 fragment from nt 2360 to the polyadenylation site at nt 4203. This cDNA, expressed from the SV40 early promoter, has been previously shown to be capable of *trans*-activating the enhancer element in the LCR of BPV-1 (43). The C59 mutants contain a TTL element within the E2 ORF at nt 2878, which inactivates BPV-1 *trans*-activation, and a TTL at nt 3881, exclusively interrupting the E5 ORF, which has no effect upon *trans*-activation (50). The data are presented as fold conversion relative to cotransfection of the HPV-16 LCR/CAT with pBR322. Quantitative analysis of similar transfections are presented in Fig. 5. ND, Not determined.

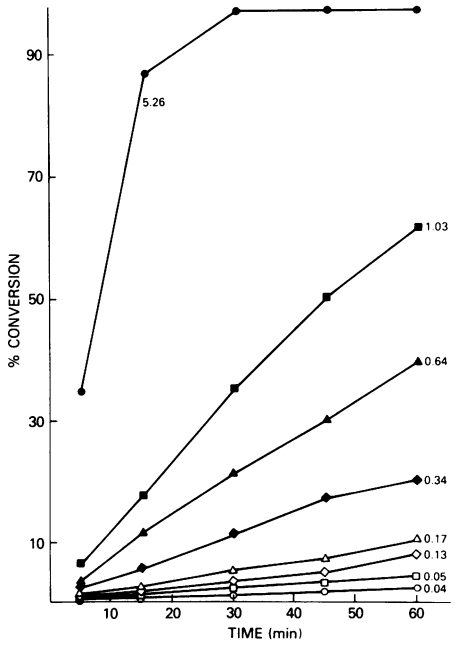

FIG. 5. Kinetic analysis of *trans*-activation. Total protein (15 μg) from the cell extracts was put into a standard CAT reaction, and samples were removed at 5, 15, 30, 45, and 60 min. The acetylated products were separated by thin-layer chromatography, and the percent acetylation was determined by liquid scintillation. The data generated, expressed as percent conversion versus time, were plotted, and regression lines were computed. The slopes of the regression lines are included in the figure and were used to assess the rate of acetylation. The different cellular extracts derived from the transfections include pSV2CAT (●), $pA_{10}CAT$ (○), p1015 plus C59 (■), p1015 plus C59-2878 (□), p1015 plus p858 (▲), p1015 plus 1028 (△), p1015 plus p859 (◆), and p1015 plus pBR322 (◇).

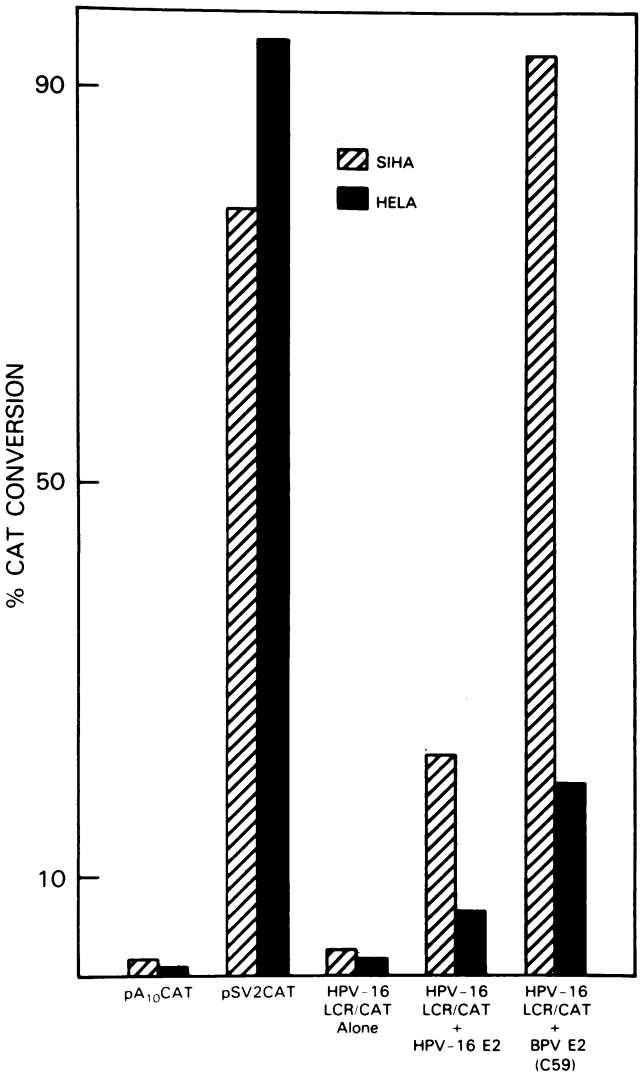

FIG. 6. Transfection of cervical carcinoma cell lines. The cervical carcinoma cell lines HeLa and SiHa were transfected by using the standard treatment of calcium phosphate precipitation, glycerol shock, and 5 mM sodium butyrate as described in Materials and Methods. Each cell line was transfected with $pA_{10}CAT$ and pSV2CAT as negative and positive controls, respectively. The HPV-16 LCR/CAT used was p863.1, the HPV-16 E2 plasmid was p858, and the BPV-1 E2 plasmid was C59. The data are presented as percent acetylation in either SiHa (▨) or HeLa (■) cells.

the levels obtained with p858. The p1013 plasmid containing the TTL at nt 4335 downstream of the E5 ORF provided the same level of *trans*-activation as the nonmutated p858 did. The insertion of the TTL into any of the three positions in the E2 ORF tested led to a complete loss of *trans*-activation function, as exhibited by p1016, p1027, and p1028. The other E2 mutants confirmed this observation. The plasmid p1026, with the frameshift in the E2 ORF at the *Bst*XI site, did not provide the *trans*-activation function nor did the mutant p1058, which is deleted of a substantial portion of the amino terminus of the E2 ORF as well as of a small part of the E4 ORF. Therefore, we conclude that the product encoded by the E2 ORF is required for *trans*-activation of the HPV-16 LCR enhancer.

BPV-1 E2 gene product could *trans*-activate the HPV-16 LCR enhancer. The E2 ORF of each of the papillomaviruses sequenced to date is well conserved at the level of the predicted amino acid structure. Therefore, it was of interest whether the E2 gene products from BPV-1 and HPV-16 could function to *trans*-activate the heterologous LCR enhancers. For these experiments, two different HPV-16 LCR/CAT constructs were used, i.e., p863.1, containing the full LCR, and p1015, which is a derivative of p863.1 containing only a segment of the LCR region from nts 7465 to 57 (Fig. 4). Each of these HPV-16 LCR/CAT plasmids could be *trans*-activated to similar levels by the HPV-16 E2 gene product, localizing the HPV-16 E2 conditional enhancer to the 3′ portion of the HPV-16 LCR (data not shown). Each of these two HPV-16 LCR/CAT plasmids could also be *trans*-activated when cotransfected with C59, a BPV-1 cDNA-containing plasmid which has been previously described and shown to express the BPV-1 E2 *trans*-activating function (43, 49). A C59 mutant with a TTL at nt 2878 in the E2 ORF eliminated this *trans*-activation function, whereas a TTL at nt 3881 in the E5 ORF had no effect. Thus, the BPV-1 E2 gene product was capable of *trans*-activating the HPV-16 LCR E2 conditional enhancer. In the reciprocal experiment, the BPV-1 LCR enhancer was also *trans*-activated by the HPV-16 E2 gene product (data not shown). Furthermore, it was shown that the minimal BPV-1 E2 conditional enhancer mapping from nts 7611 to 7804 [B. A. Spalholz, C. C. Baker, P. F. Lambert, and P. M. Howley, Cancer Cells (Cold Spring Harbor), in press] could also be *trans*-activated by the HPV-16 E2 gene product, indicating that the sites of activity for both E2 gene products are likely to be the same (data not shown).

Relative levels of *trans*-activation. To examine the relative levels of *trans*-activation provided by the BPV-1 and HPV-16 E2 gene products, a kinetic analysis was performed using the HPV-16 LCR/CAT plasmid p1015 (Fig. 5). Although the absolute levels of *trans*-activating proteins are unknown, the amount of plasmid DNA required to provide a maximum level of *trans*-activation was determined for p858, p859, and C59 (data not shown). Thus, the kinetic analyses (Fig. 5) were performed with plasmid concentrations which produced saturating *trans*-activation levels. Each of the acetylation reactions was normalized for the amount of total protein (15 μg) from the cell extracts and was carried out for the various times shown (Fig. 5). From these data, a regression line was computed and the slopes were determined as a measure of the relative reaction rates. The linear slopes measured as percent chloramphenicol acetylated per minute are indicated in Fig. 5. The highest level of *trans*-activation of the HPV-16 enhancer was provided by the BPV-1 E2-expressing plasmid C59. *trans*-activation by the HPV-16 E2 gene from p858 provided only about two-thirds of the activity provided by BPV-1 E2. In general agreement with data presented in Fig. 3, the shorter HPV-16 E2 DNA clone p859 was only about 50% as active as p858. Finally, mutations in the E2 genes of either HPV-16 (p1028) or BPV-1 (C59-2878) resulted in the elimination of *trans*-activation functions, reducing the conversion rates to that obtained with $pA_{10}CAT$ alone or with HPV-16 LCR/CAT cotransfection with pBR322.

Assay for E2 activity in cervical carcinoma cell lines. Recently, several continuous cell lines derived from human cervical carcinomas have been found to contain and express HPV-16 and HPV-18 sequences (3, 5, 33, 37, 38, 51). We were interested in whether such cell lines express a factor which would *trans*-activate the HPV-16 LCR E2 conditional enhancer in the absence of an added E2- expressing plasmid.

Two cell lines were examined, i.e., HeLa cells which contain 10 to 50 integrated copies of HPV-18 DNA (38, 51) and SiHa cells which contain a single integrated copy of HPV-16 (3, 51). We first examined whether the HPV-16 LCR/CAT plasmid (p863.1) transfected alone into HeLa or SiHa cells would express CAT. No activity above that obtained with $pA_{10}CAT$ alone was detected in either of these cell lines (Fig. 6). Thus, neither of the cell lines expressed a viral or cellular gene product which could *trans*-activate the LCR conditional enhancer. Furthermore, cotransfection of either the HPV-16 E2 (p858) or the BPV-1 E2 (C59) resulted in *trans*-activation of p863.1, indicating that these cells did not harbor a factor which specifically blocked *trans*-activation of the LCR enhancement of the SV40 early promoter.

DISCUSSION

These studies were carried out as an extension of our interest in the transcriptional regulation of papillomaviruses. *trans*-activation of an LCR conditional enhancer by the BPV-1 E2 gene product has already been described by this laboratory (43, 50). We were interested in determining whether E2 *trans*-activation was a regulatory phenomenon peculiar to BPV-1 or a general control feature of all papillomaviruses. The data presented in this study clearly demonstrate that, as with BPV-1, a transcriptional enhancer element exists within the LCR of HPV-16 and that this element is a conditional enhancer responsive to the viral E2 gene product.

The primer extension analysis depicted in Fig. 2 demonstrated that the increase in CAT activity associated with *trans*-activation of the HPV-16 LCR enhancer was due to an increase in the steady-state level of CAT-specific mRNA. Similar experiments have confirmed that the mechanism of action for BPV-1 E2-mediated *trans*-activation of its homologous LCR enhancer is transcriptional and is due to an increase in levels of CAT RNA (B. A. Spalholz, P. F. Lambert, C. L. Yee, and P. M. Howley, J. Virol., in press). For HPV-16 as well as for BPV-1, the increase of steady-state levels of CAT mRNA is presumed to be due to an increase in the rate of transcription, since the target for this *trans*-activation is not part of the CAT RNA, making it unlikely that the effect is due to specific RNA stability.

Genetic dissection of the 3′ early ORFs established that the integrity of the E2 ORF was required for functional *trans*-activation of the LCR enhancer element of HPV-16. This is in agreement with analogous studies which have localized the *trans*-activation function of BPV-1 to the E2 ORF (43, 50). In addition, we showed that the E2 *trans*-activation functions of BPV-1 and HPV-16 could operate on the heterologous LCR enhancer elements. The E2 gene products predicted for the sequenced papillomaviruses contain domains which appear to be well conserved at the level of amino acid sequence. Recent experiments defining the minimal conditional enhancer of BPV-1 indicate that the repeated motif $ACCGN_4CGGT$, a sequence characteristic of all papillomaviruses sequenced to date (9), is a functionally necessary component of this minimal enhancer (Spalholz et al., in press). Recent data from two independent laboratories have demonstrated that an E2 protein expressed in bacteria is capable of specific binding to these motifs (2; C. Moskaluk and D. Bastia, Proc. Natl. Acad. Sci. USA, in press). This repeated element occurs at three positions in HPV-16, i.e., at nts 7450, 35, and 50. The smaller HPV-16 LCR/CAT plasmid p1015 contains sequences from nts 7467 to 57 and thus contains only one complete copy of these three motifs (the motif at nt 50 was partially disrupted by cloning at nt 57). Since this plasmid was fully responsive, the motif at nt 7450 and at least part of the sequence at nt 50 was expendable for *trans*-activation under the experimental conditions used.

The HPV-16 LCR/CAT plasmids were used to determine whether cellular factors with E2-like activity were present and expressed in the HPV DNA-positive human cervical carcinoma cell lines HeLa and SiHa. Recent data concerning the structure of the integrated HPV-16 and HPV-18 sequences in these cell lines (3, 38) have demonstrated that the E2 ORF of the integrated viral DNAs is disrupted in each of these lines. It is possible, however, that a functionally analogous cellular factor is provided by these cells. The experiments presented in Fig. 6 indicated that neither a viral E2 nor a cellular E2-like activity was present in these cells, since the HPV-16 LCR/CAT plasmid was not active in either of these cell lines in the absence of an exogenously added plasmid expressing the E2 gene. In similar experiments conducted with the CaSki cell line, another cervical carcinoma cell line containing >600 copies of integrated HPV-16 (51), no *trans*-activation of the HPV-16 LCR/CAT was noted, although the transfection efficiency of this cell line was very low (data not shown). Recent analyses of HPV-16 transcription in the SiHa and CaSki cell lines have indicated that the majority of the RNA transcripts are derived from the E6, E7, and E1 ORFs (3, 40). In BPV-1, the promoters upstream of the E6 ORF (P_{7940} and P_{89}) have been shown to be responsive to the E2 conditional enhancer (Spalholz et al., in press). It seems likely that the analogous promoters in HPV-16 would also be responsive to the E2 conditional enhancer. Thus, in the absence of viral or cellular factors to activate the E2-responsive LCR enhancers in these cervical carcinoma cell lines, transcription from the promoters upstream of E6 and E7 may be owing to a basal level of expression, indicating that E2 *trans*-activation may not be absolutely required for expression from these promoters. Alternatively, integration may have occurred in the host chromosome in the vicinity of a functional cellular enhancer, thereby obviating the requirement for E2 induction of the integrated viral promoters.

It is interesting that integration of the HPV DNA in these cell lines, as well as in several tissue samples derived from human cervical carcinomas, appears to occur preferentially within the E2 ORF (3, 38). It is possible that carcinogenic progression is directly related to the integration event and the resulting inactivation of the E2 genes. This could result in the release of the promoters directing synthesis of the E6 and E7 RNAs from the regulated control of the virus. Preliminary studies have indicated that in addition to a transcriptional activation function, the 3′ portion of the BPV-1 E2 gene may encode a factor which negatively regulates expression from the BPV-1 LCR enhancer [P. F. Lambert, B. A. Spalholz, and P. M. Howley, Cancer Cells (Cold Spring Harbor), in press]. Thus, in addition to the inactivation of a transcriptional *trans*-activator, the disruption of the E2 ORF by integration in cervical carcinomas may also inactivate a transcriptional repressor. Experiments are currently being conducted to assess whether HPV-16 encodes a transcriptional repressor mapping to this portion of the genome.

Enhancers are known to regulate a variety of viral and cellular transcription units (24). They are *cis*-dependent control elements which augment transcription of linked genes in a relatively orientation- and position-independent manner. Some enhancer elements have been shown to be

tissue- and species-specific (10, 11, 25, 35, 45), presumably relying on cellular factors mediating control of transcription. Many eucaryotic viral systems have been shown to encode genes which act in *trans* to regulate viral gene expression. The SV40 late promoter is *trans*-activated by large T antigen (1, 6, 21), the adenovirus E2 and E3 genes are *trans*-activated by the E1a gene product (22, 23), the herpesvirus delayed-early genes are *trans*-activated by immediate-early gene products (31), and the human T cell lymphotropic virus types I and II LTR enhancer elements are *trans*-activated by their respective *tat* proteins (42). The LTR of the human immunodeficiency virus also contains a *cis*-responsive element which can be *trans*-activated by the bipartite *tat* gene product, and at least one part of this *trans*-activation is at the level of transcription (8, 14, 41). The activation of viral enhancer elements by specific viral gene products may be analogous in some cases to the induction of cellular transcription units during normal differentiation, development, or hormonal response. In each of these instances, specific factors are believed to modulate the transcriptional activity of particular operons. It is clear that E2-mediated *trans*-activation of the viral LCR is only one facet of the papillomavirus life cycle. It is feasible that the E2 gene may act as an immediate-early gene whose product(s) is required for activation of early viral promoters proximal to the LCR enhancer. Furthermore, the E2 gene may express, in addition to an activating function, a transcriptional repressor. We believe that a clear definition of these regulatory functions and pathways is vital to understanding the potential role of the papillomaviruses in carcinogenesis.

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