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The E6/E7 Promoter of Human Papillomavirus Type 16 Is Activated in the Absence of E2 Proteins by a Sequence-Aberrant Sp1 Distal Element

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The E6/E7 promoter of all genital human papillomaviruses is responsible for expression of the viral transforming genes. Centered 60 bp upstream of the transcription start, it contains a 20-bp segment with partially overlapping binding sites for the viral E2 proteins and for a cellular factor that was identified by footprint experiments. Bandshifts, bandshift competitions, and footprints revealed that protein complexes between nuclear extracts and these sequences have binding properties indistinguishable from those of the Sp1 factor that binds the simian virus 40 early promoter GC motif. Reactions of these complexes with anti-Sp1 antiserum were analyzed by superbandshifts and precipitation with protein A, and the results confirmed the identity of this transcription factor as Sp1. Sp1 binds in simian virus 40 and different human papillomavirus promoters the consensus sequence 5′-NGGNGN-3′. RNase protection analysis of in vitro or in vivo transcriptions with wild-type and mutant test vectors shows that the E6/E7 promoter of human papillomavirus type 16 is functionally dependent on the Sp1 distal promoter element. In all genital papillomaviruses, the Sp1 hexamer is invariably spaced by a single nucleotide from the distal E2 element, suggesting some precise interaction between Sp1 and E2 proteins. Published experimental evidence documents negative regulation of the E6/E7 promoter by E2 proteins through the proximal E2 element, whereas only minor quantitative differences in E6/E7 promoter function after cotransfection with E2 expression vectors were observed in this study. A detailed study of the interactions of Sp1 and E2 proteins with one another and with the corresponding three binding sites may reveal a complex modulation of this promoter.

Human papillomaviruses (HPVs) that infect the genital tract, in particular HPV type 16 (HPV-16), play a central role in the etiology of a variety of epithelial tumors such as cervical carcinomas (for a review, see reference 47). It is therefore of paramount interest to understand the life cycle and gene expression of HPVs, particularly since several molecular and epidemiological studies have suggested changes of papillomavirus transcription as cofactors of the transformation process (4, 6, 7, 21, 37, 48, 49).

Papillomaviruses have a circular genome of about 7,900 bp. Roughly 90% of this DNA codes for proteins, and 10%, referred to as the long control region, contains a large fraction of the *cis* regulatory elements (30, 47). A promoter whose position defines the 3′ border of the long control region is of particular interest, since transcripts starting at this position encode transforming proteins, namely, E6 and E7 (18, 45, 51). In the HPVs which are responsible for benign and malignant lesions of the genital tract, the E6/E7 promoter is under the influence of several cooperating or antagonistic regulatory elements. HPV-16, the prototype of this group, has upstream of its E6/E7 promoter P97 (45) a TATA box (at position −32 to −26 relative to P97) and two palindromic E2 protein-binding sites (at positions −36 to −47 and −51 to −62 relative to P97). We have previously observed that a nuclear protein binds to a sequence that partially overlaps with the distal E2-binding site and termed this footprint fp2u (23) (Fig. 1). A 400-bp fragment with cell type-specific enhancer activity is centered 350 bp upstream of P97 (14, 22) and contains binding sites for the transcription factors NF1, AP1, PVF, and the progesterone and glucocorticoid receptors (6, 10, 24). This composition of enhancer and promoter elements is typical for all genital papillomaviruses but diverges from those of HPV-1 and HPV-8 (24), which infect cutaneous epithelia. For bovine papillomavirus type 1 (BPV-1), sequence inspection also suggests quite a different composition of transcription factor-binding sites (our unpublished observations). In BPV-1, transcription is activated by the large form of the viral E2 proteins binding to clusters of E2-binding sites. This effect is annihilated by short forms of the protein (for a review, see reference 31). The mechanism differs in genital HPVs, in which E2-binding sites overlap with potential promoter elements (Fig. 1), a sequence organization that permits negative regulation even through the large E2 protein (5, 39, 48).

Our research aims to expand the understanding of the regulation of gene expression of HPV-16 by documentation of the functional contribution of each transcription element. This study documents that the E6/E7 promoter depended on a distal element. The element is bound by the transcription factor Sp1, although sequence comparison of the element with the genomes of six HPV types shows only a 3-bp consensus with the classical Sp1 GC box of several viral and cellular promoters (17, 29). The Sp1 footprint fp2u overlaps with one binding site for the E2 protein, and the hexameric Sp1 recognition sequence in all genital papillomaviruses is spaced by exactly 1 bp from the ACC(N_6)GGT recognized by E2 proteins, a strict sequence alignment that makes interaction between Sp1 and E2 likely.

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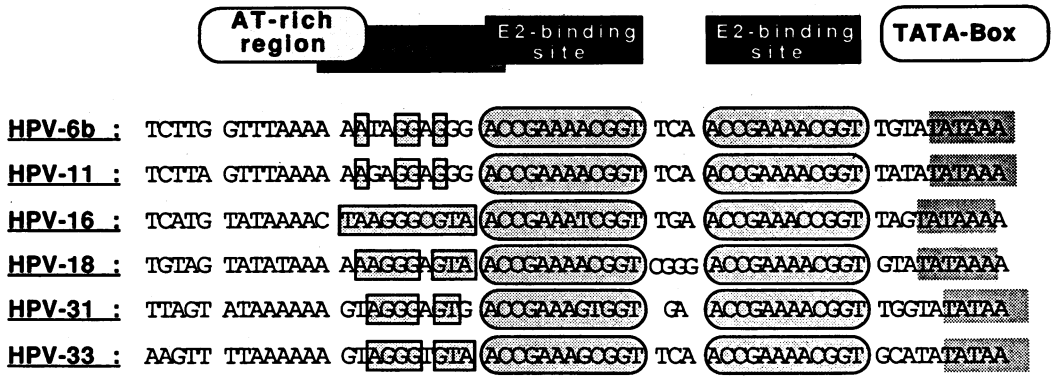

FIG. 1. Sequence comparison between the TATA box of the E6/E7 promoter (shaded) and a second AT-rich region located approximately 50 bp upstream of six different HPVs. In HPV-16, this region contains a protein-binding site termed fp2u which is shown in this paper to bind the Sp1 transcription factor. Nucleotides within this Sp1-binding site that are identical to the HPV-16 sequence (shaded boxes) and the E2-binding sites (shaded ovals) are shown. The spacing of the elements within the promoters of all six HPVs is well conserved. The documented or presumptive E6/E7 promoter segments are from published sequences (12, 13, 15, 25, 42, 44).

MATERIALS AND METHODS

Construction of plasmids. The basic vectors OVEC 1, SV40-OVEC, and OVEC-REF were constructed in the laboratory of Walter Schaffner (52), who made them available to us. The constructs HPVpOVEC and HPVp2u*OVEC contained in the *SalI-PstI*-digested OVEC 1 vector a 79-bp oligonucleotide with the sequence 5'-TCGACCCGGG TATAAAACTAAGGGCGTAACCGAAATCGGTTGAAC CGAAACCGGTTAGTATAAAAGCAGACATTCTGCA-3' which includes the genomic positions 16 to 80 of HPV-16 (44) such that the HPV-16 TATA box replaces the original β-globin TATA box with unaltered spacing to the β-globin transcription start. To eliminate binding of the fp2u factor in the 2u* mutant, we inserted the sequence 5'-TCGAC CCGGGTATAAAACTAAtttaGTAACCGAAATCGGTTG AACCGAAACCGGTTAGTATAAAAGCAGACATTCT GCA-3'. To derive the vectors SVeHPVpOVEC and SVeHPVp2u*OVEC from these two constructs, a 172-bp simian virus 40 (SV40) fragment with two 72-bp enhancer repeats previously subcloned as an *SphI* dimer from pSV2CAT into pUC19 was inserted between the respective *SalI* and *SmaI* sites of HPVpOVEC and HPVp2u*OVEC (Fig. 2). Similarly, to obtain HPVe/pOVEC and HPVe/p2u*OVEC, a 400-bp fragment with the enhancer of HPV-16 (22) was inserted with *SalI* linkers into the two promoter vectors. The BPV-1 E2 expression vector pC59 was a kind gift of P. Howley (38). To monitor positive regulation by E2 protein, we cloned the sequence 5'-ACCGAAATCGGTT GAACCGAAACCGGT-3' with *SalI*-protruding ends into pBLCAT2 (34), a vector that has a polylinker upstream of a herpes simplex virus-thymidine kinase promoter fused to the chloramphenicol acetyltransferase (CAT) gene.

Cell culture and transfection procedures. HeLa, SiHa, and CaSki cells were grown in minimal essential medium supplemented with 10% fetal calf serum to less than 80% confluency, trypsinized, and washed twice with phosphate-buffered saline. Cells (3×10^6) in 600 μl of phosphate-buffered saline were mixed with 15 μg of reporter plasmid and 2 μg of OVEC-REF in 200 μl of phosphate-buffered saline and left for 10 min on ice before electroporation with a Bio-Rad Gene Pulser with a capacitance extender at 960 μF and 250 V. After electroporation, 1.6 ml of cells was plated into 30 ml of medium in 150-mm petri dishes. Harvesting for RNA analysis was done after 48 h without any medium changes. pC59 and pBLCAT2-E2 were cotransfected at 20 μg each; plates without the E2 expression vector received the same amount of pUC19 instead of pC59.

RNA extraction and analysis. Transfected cells were washed three times with phosphate-buffered saline before 5 ml of guanidinium mix (50% [wt/vol] guanidinium-thiocyanate, 50 mM Tris hydrochloride [pH 7.5], 20 mM EDTA, 2% *N*-laurylsarcosine, 150 mM β-mercaptoethanol) per 150-mm dish was added. The cell suspension was transferred into a

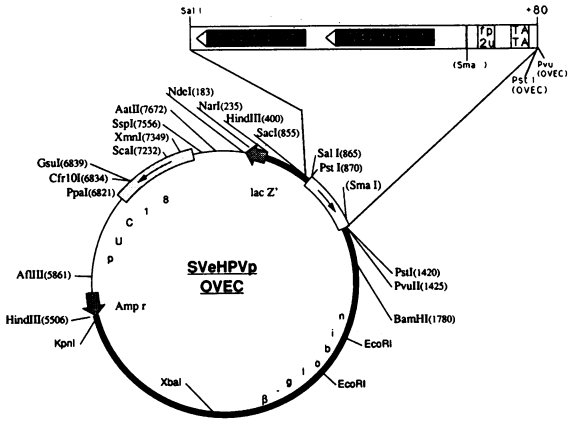

FIG. 2. A map of one of the OVEC constructs representing the vectors used in this study. The plasmid consists of three segments, the pUC18 portion, the β-globin portion, and the enhancer-promoter portion (enlarged segment). The latter was inserted into the β-globin gene as a *SalI-PstI* fragment, thereby replacing the β-globin TATA box (which has the sequence CATA) with the HPV-16 TATA box. In this particular construct, the SV40 enhancer was cloned as an *SphI* 72-bp repeat into pUC18, from where it was retrieved as a *SalI-HindIII* (blunt ended) fragment for reinsertion into an OVEC vector with an HPV-16 oligonucleotide representing P97 promoter sequences (see Materials and Methods). The HPV-16 400-bp enhancer (23) was cloned with *SalI* linkers into the same oligonucleotide. The two constructs brought enhancer elements of SV40 within 10 bp of fp2u, whereas the distance between the HPV-16 enhancer (23) and fp2u exceeded 100 bp. Major restriction sites are given as landmarks, but the map is not comprehensive, since parts of the β-globin reporter gene were not sequenced. In the case of SV40-OVEC only the two 72-bp repeats were cloned as a *SalI* fragment upstream of the β-globin TATA box.

50-ml Falcon tube containing 2 g of CsCl, mixed, and layered on top of a 4-ml cushion of 5.7 M CsCl in 12-ml ultracentrifuge tubes. The tubes were spun in an SW41Ti rotor at a speed of 30,000 rpm for 22 h. The RNA pellet was dissolved in 400 μl of H_2O, DNase I digested, phenol-chloroform extracted, and precipitated with ethanol. After photometric determination of the RNA, 10 μg of RNA was used to hybridize a [α-^{32}P]UTP-labeled SP6 polymerase-synthesized RNA probe, covering the sequence of OVEC 1 from position −37 (*Sal*I site) to position +179 (*Taq*I site). This hybridization and all subsequent steps were performed as previously described (1). The design of probe and test vectors was such that the HPV-16 or β-globin promoter in test vectors gave an RNA signal of 179 nucleotides (Fig. 5 and 6, bands ct), whereas the transcript of the reference vectors after processing with the same probe gave a signal at 160 nucleotides (Fig. 5 and 6, bands ref). CAT transcripts originating from pBLCAT2-E2 were processed to give a 210-nucleotide signal by use of an SP6-CAT vector containing the inverted *Eco*RI-*Pvu*II fragment from pBLCAT2 (35).

In vitro transcription. The 10-μl reaction mixtures contained 500 ng of test plasmid, 200 ng of OVEC-REF, 5 μl of nuclear extract (corresponding to 15 μg of protein [16, 52]), and in vitro transcription buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.9], 10% glycerol, 20 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol, 5 mM creatine phosphate, 0.5 mM each ATP, UTP, GTP, and CTP). The samples were incubated at 30°C for 60 min. Subsequently 10 μl of a solution containing 8 M urea, 2% sodium dodecyl sulfate, and 20 mM EDTA was added. The samples were boiled for 3 min prior to phenol-chloroform extraction and ethanol precipitation. After a thorough DNase I digestion to remove all the template DNA, the RNA was analyzed as described above for RNA from transfected cells.

Footprint and bandshift procedures. The 10-μl reaction mixtures for the footprint assay contained 5 μl of nuclear extract (corresponding to 15 μg of protein [16, 53]), 3 μl of SMK mix (12 mM spermidine, 12 mM $MgCl_2$, 120 mM KCl), 100 ng of poly(dI-dC), and 5,000 cpm (ca. 2 ng) of end-labeled DNA test fragment, and competitor oligonucleotide (approximately 100-fold excess) was added where indicated. After preincubation of the mixture in the absence of the test fragment for 15 min on ice, the labeled DNA was added and incubation was continued for another 15 min on ice. Subsequently the samples were partially digested at 25°C with DNase I by adding 2 μl of DNase I mix (17 mM $MgCl_2$, 238 μg of calf thymus DNA per ml, 70 to 290 ng of DNase I per ml; Cooper Biomedical) for 90 s, which was followed by DNA extraction with phenol-chloroform and ethanol precipitation. The DNA was suspended in formamide loading buffer (80% formamide, 1 mM EDTA, 5 mM NaOH, 0.05% bromophenol blue–xylene-cyanol) and analyzed on 6% denaturing polyacrylamide–urea gels.

For the bandshift assay the incubation mixture and incubation condition were essentially the same but with only one-fifth of the amount of nuclear protein. The labeled DNA in the bandshift assay was a double-stranded oligonucleotide labeled with Klenow polymerase. The sequence of the HPV-16 fp2u oligonucleotide was 5′-TGCACTAAGGGCG TAACCGAAATCTGCA-3′. The sequence of the oligonucleotide from an SV40 Sp1-binding site was 5′-TGCACG ATGGGCGGAGTTAGGGGCTGCA-3′. The following double-stranded oligonucleotides were used as heterologous competitors: fp2u*, 5′-TGCACTAACATGCTAGAACTCG TCTGCA-3′; HPV-6/11 fp2u, 5′-TCGACAAGAGGAGGG ACCGAAAACTGCA-3′. The oligonucleotide-protein complexes were separated on a 1-h prerun 5% polyacrylamide gel (60:1 bisacrylamide) containing 2.5% glycerol in a solution containing 50 mM Tris base, 380 mM glycine, and 2 mM EDTA. After electrophoresis at 12 mA constant current (150 to 130 V, 0.75-mm-thick gel, 15 by 14 cm) for 2 h, the gel was fixed in 10% acetic acid for 10 min and then dried and exposed to Kodak X-AR film. For superbandshifts, the nuclear extracts were preincubated for 1 h on ice with 2 μl of reconstituted preimmune or anti-human Sp1 antiserum per reaction. This preparation was a kind gift of R. Tjian and S. P. Jackson, University of California, Berkeley.

RESULTS

Sp1 binds to an aberrant Sp1 recognition sequence at the E6/E7 promoter of genital HPVs. DNase I protection analysis has revealed a footprint termed fp2u in the long control region of HPV-16 (23). fp2u is centered 65 bp 5′ of the E6/E7 promoter P97. Sequence comparison between six different genital HPVs revealed homologies with this segment, which is immediately upstream of and overlapping with the distal E2-binding site, suggesting an element involved in promoter function (Fig. 1). In HPV-16, the hexamer GGGCGT shows sequence relationship to published Sp1-binding sites GGG CGG, such as those of the SV40 early promoter, the herpes simplex virus-thymidine kinase promoter, and the hsp70 gene promoter (17, 29, 36). In other genital papillomaviruses this sequence is quite divergent, e.g., the motif AGGAGG in HPV-6 and -11. Also, adenine residues rather than guanine residues in positions 7 and 8 following these six bases have been found to reduce the affinity for Sp1 (29), which leaves doubts about the nature of this transcription factor-binding site.

To clarify the identity of this element, we performed bandshift experiments with the SV40, HPV-16, and HPV-6 sequences. Two apparently identical bands were obtained with nuclear extracts from HeLa cells, using the SV40 or the HPV-16 motif (Fig. 3). The bands were competed with homologous as well as with the respective heterologous oligonucleotide and even with an oligonucleotide representing the strongly aberrant HPV-6/11 motif but not with a mutated sequence. Competition was somewhat less by the HPV-16 motif than by the SV40 motif and was weakest by the HPV-6/11 motif. This is likely to reflect a gradient of affinities, as suggested also by the weaker but qualitatively identical bandshift of the HPV-6/11 motif (data not shown). We conclude that protein complexes with identical binding specificities and electrophoretic behavior formed on the SV40 GC motif, on HPV-16 fp2u, and on the corresponding HPV-6/11 motif.

To further clarify the nature of these complexes, we preincubated the nuclear extracts prior to the binding reaction with a rabbit serum raised against human Sp1 protein, which was expressed in *Escherichia coli* (S. P. Jackson and R. Tjian, personal communication). This reaction led to a new complex in a bandshift experiment that barely entered the gel, whereas complex II of the bandshift was strongly diminished and complex I was slightly diminished, both with SV40 GC and HPV-16 fp2u (Fig. 3, lanes 11 to 14). No change was observed after preincubation with a preimmune serum. In addition, both complexes were selectively precipitated through addition of protein A-Sepharose beads to the nuclear extract-antiserum mix (Fig. 3, lanes 15 and 16). We conclude that Sp1 factor identified both by binding speci-

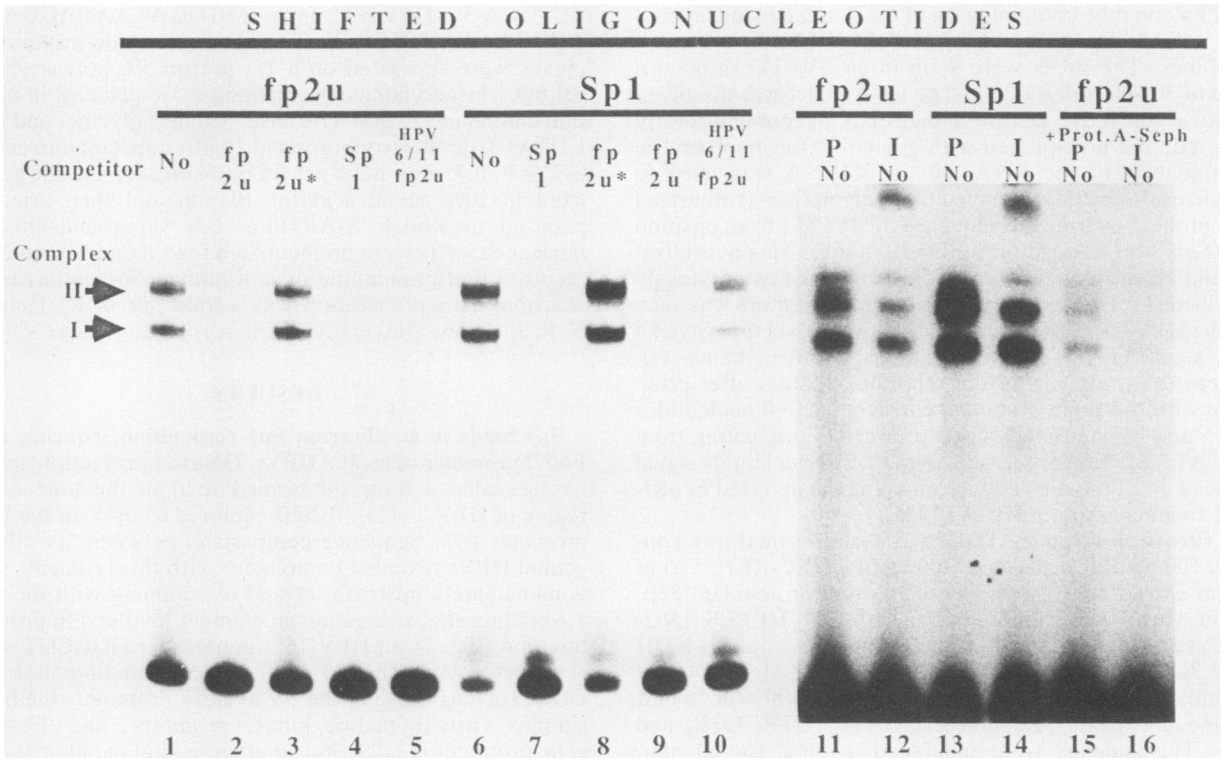

FIG. 3. Bandshift experiment with radiolabeled oligonucleotides comprising either the fp2u site of HPV-16 or an Sp1 site of SV40. Nuclear extract from HeLa cells was incubated with the fp2u oligonucleotide (lanes 1 through 5) or an Sp1 oligonucleotide derived from the SV40 promoter (lanes 6 through 10) and run through a native polyacrylamide gel. Two specific oligonucleotide-protein complexes (Complex I and Complex II) could be efficiently competed with on addition of homologous (lanes 2 and 7) and heterologous (lanes 4, 5, 9, and 10) unlabeled oligonucleotides, including the fp2u homologies of HPV-6 and HPV-11. The complexes could not be competed with a mutated (fp2u*) oligonucleotide (lanes 3 and 8). Preincubation of the nuclear extract with anti-Sp1 antiserum led to a superbandshift, weakening in particular the signal of complex II (lanes 12 and 14), whereas no additional shift occurred under the influence of the preimmune serum (lanes 11 and 13). Incubation of the nuclear extract-antiserum mix with protein A-Sepharose beads (Pharmacia) nearly eliminated complexes I and II (lanes 15 and 16).

ficity and by antigenic properties bound to the HPV-16 motif.

CaSki nuclear extracts lead to Sp1 but not to E2 footprints on the P97 promoter. To analyze further the nature of protein-DNA complexes on the HPV-16 promoter sequence, we performed a footprint competition experiment with nuclear extracts derived from the CaSki cell line. This experiment was done to understand whether the bandshift competition observed in Fig. 3 corresponded to the disappearance of a specific fp2u footprint. We also hoped to visualize factors other than Sp1 on this DNA segment, in particular the E2 protein that is proposed to be expressed in CaSki cells from endogenous HPV-16 genomic copies (32).

A single footprint, fp2u, can be seen on a segment of approximately 60 bp centered 70 bp upstream of P97 (Fig. 4). This footprint disappeared after addition of excess fp2u oligonucleotide. The distal E2-binding site was not protected, not even after competition for the bound Sp1 factor, which speculatively could have displaced E2 protein from binding. We conclude that Sp1 bound to fp2u was the only cellular factor which bound to distal P97 promoter sequences and that the affinity of E2 proteins or their concentrations in CaSki cells were insufficient for detection in this assay.

The HPV-16 P97 promoter is active in an in vitro transcription reaction and stimulated by the Sp1-binding site. The position of the Sp1-binding site relative to the E6/E7 transcriptional start is suggestive of a function as a distal promoter element, i.e., stimulation of transcription through the proximal promoter elements, particularly in response to an enhancer. To test for this property of fp2u, we performed in vitro transcription and in vivo transfection experiments with OVEC vectors.

OVEC vectors are designed to measure the transcription of the 5′ segment of a human β-globin gene through an RNase protection assay (52). In a typical experiment, the enhancer-dependent activation of a promoter is determined with two constructs, one with and one without a distal promoter element but both carrying a TATA box (52). This test system is superior to a CAT assay (26), since differences in transfection efficiency can be identified with the help of a cotransfected vector that gives rise to a reference transcript of different length. Also, these vectors allow monitoring of particular transcription start sites rather than lumping transcripts from different starts into one signal.

We constructed two vectors (Fig. 2) that contained a 65-bp HPV-16 segment encompassing the promoter elements (Fig. 1) approximately 10 bp downstream of the 3′ border of a tandem repeat of the 72-bp enhancer of SV40. SVeH PVpOVEC carried the wild-type sequence, whereas SVeHPVp2u*OVEC carried a mutated Sp1-binding site.

In vitro transcription with nuclear extracts from SiHa cells demonstrated that the P97 transcript could be obtained in vitro (Fig. 5). The band was weaker in the mutant than in the wild type, indicating a functional contribution of the Sp1-

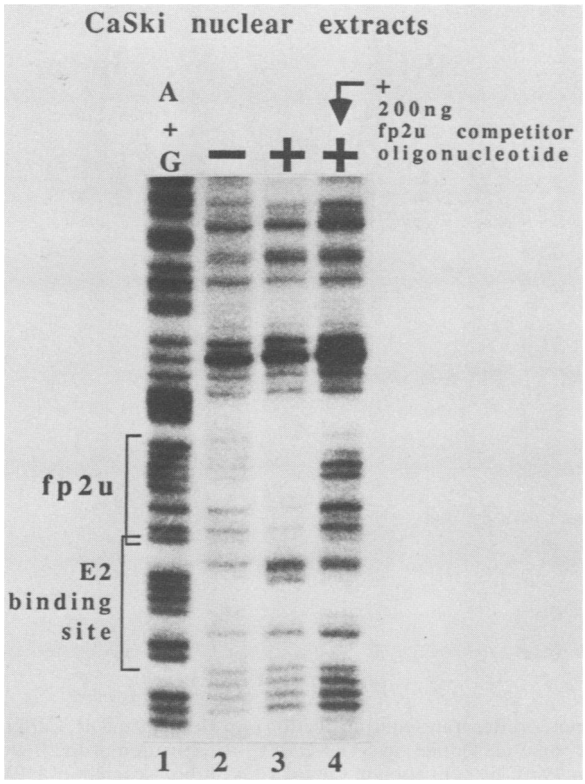

FIG. 4. Footprint experiment of the promoter region of HPV-16 with CaSki nuclear extracts. Lane 1, A+G Maxam & Gilbert cleavage products of the asymmetrically labeled DNA fragment; lane 2, partial DNase I digest of the labeled DNA fragment in the absence of nuclear proteins; lane 3, protection of the Sp1 site by nuclear proteins (indicated with a bracket labeled fp2u); lane 4, footprint reaction in the presence of an unlabeled excess of fp2u oligonucleotide, depriving the nuclear extract from Sp1 proteins that induce fp2u. Whereas protection of bands in the fp2u region disappeared, the E2-binding site remained unprotected, most likely because of a low concentration or absence of E2 protein in CaSki cells.

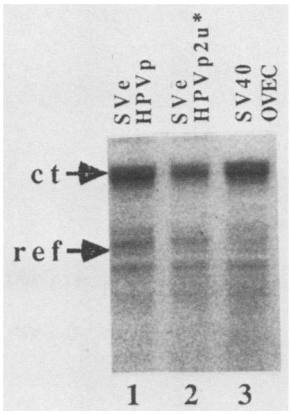

FIG. 5. Effects of in vitro transcription with SiHa nuclear extracts on HPV-16 P97 promoter activity independence of an Sp1-binding site. The in vitro-generated mRNA was assayed by SP6 RNase mapping. All three templates showed correct transcription (ct) initiation of the β-globin reporter gene from the HPV-16 promoter P97 or from the β-globin promoter. A reference start coming from a plasmid template (ref) was added to monitor equal transcription efficiency. Lane 1, Correct transcripts which were initiated from SVeHPVpOVEC, a vector with wild-type promoter sequences; lane 2, fp2u mutation reduction of the transcript; lane 3, transcripts from SV40-OVEC, a template without HPV-16 sequences but with SV40 enhancer elements in a promoter configuration activating the β-globin TATA box (48).

binding site to this signal. P97 was not strictly dependent on Sp1 function, since the close positioning of the SV40 enhancer in promoter configuration in this particular vector series was sufficient to give some activation of a TATA box in the absence of a distal promoter element (52). This was confirmed by in vitro transcription from the corresponding vector SV40-OVEC (Fig. 5, lane 3), which contained the β-globin TATA box but no additional promoter element.

The Sp1-binding site of HPV-16 activates P97 in vivo. To test the function of the Sp1 distal element in vivo, we transfected OVEC vectors with wild-type and mutated HPV-16 Sp1-binding sites and the SV40 enhancer in close position into SiHa, CaSki, and HeLa cells. Comparison of the signal (Fig. 6, lanes 2 and 3, 5 and 6, and 9 and 10, bands ct) documented partial loss of function in all three cell lines through mutation of the Sp1-binding site. The residual activity is due to the close position of the SV40 enhancer to the TATA box (52).

These experiments were performed with three different cervical carcinoma cell lines, HeLa, SiHa, and CaSki. The cell lines all contain chromosomally integrated HPV DNA, namely, HPV-18 in HeLa cells and HPV-16 in SiHa and CaSki cells. HeLa and SiHa cells contain the HPV genomes integrated in such a way that interruption of the E2 gene eliminates its expression. In contrast, CaSki contains some HPV-16 genomic copies without interruption of E2 (3) and has been proposed to express E2 protein (32), although the existence of an mRNA giving rise to a full-length E2 protein is disputed (45). The full-length E2 proteins of HPV-16 and BPV-1 have been shown to stimulate transcription, when their target sequences are positioned relative to the promoter in enhancer configuration, i.e., in remote position. However, E2 proteins can down-regulate the authentic HPV-16 and HPV-18 E6/E7 promoters because of the overlap of their binding sites with promoter elements (5, 39, 48). Surprisingly, an expected modified transcription in CaSki cells was not observed. From this observation it may be suspected that concentrations of E2 protein in CaSki cells were insufficient to regulate the supposedly large number of transfected promoter copies.

These observations suggested a further experiment to address two questions: first, the influence of the large E2 protein on our promoter construct and test conditions, and second, the interaction of the natural HPV-16 enhancer with P97 promoter elements. The plasmids HPVe/pOVEC and HPVe/p2u*OVEC are identical to the corresponding OVEC vectors with the SV40 enhancer but carry the HPV-16 400-bp enhancer instead of the SV40 enhancer. Besides bringing together the homologous transcriptional elements, they also create an alignment for a more stringent test of the distal element properties of fp2u, since the important enhancer elements are in the center of the 400-bp fragment (10) and are thus more than 100 bp away from the Sp1 site, in contrast to SVeHPVpOVEC, in which this distance is only 10 bp. To test for the influence of an E2 protein on the wild-type promoter in the presence of an internal control, we transfected HeLa cells with HPVe/pOVEC with and without the BPV E2 expression vector pC59 (38) and with a construct termed pBLCAT2-E2, which contains the E2-binding sites of the HPV-16 promoter in enhancer configuration

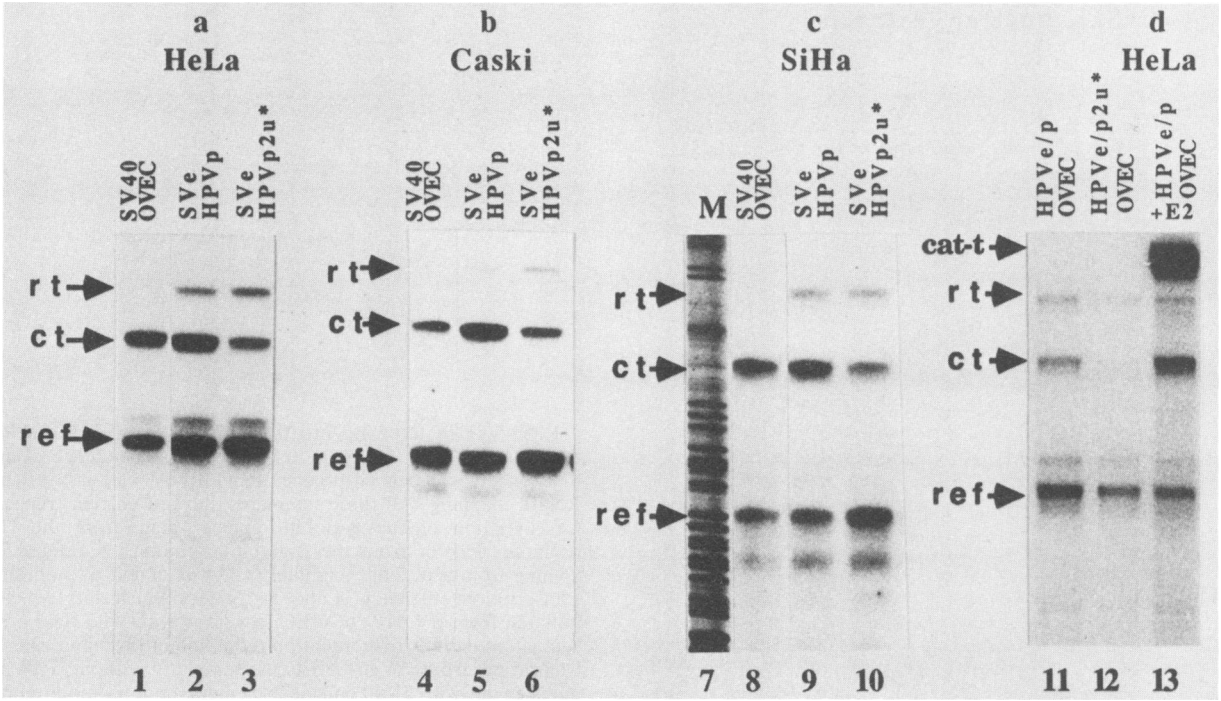

FIG. 6. In vivo transcription from wild-type and mutant HPV-16 P97 promoters after transient transfection into HeLa (a and d), CaSki (b), and SiHa (c) cells. The correct transcript (ct) initiates in each vector at the same nucleotide, in SV40-OVEC by activation of the β-globin TATA box through the SV40 enhancer, and in SVeHPVpOVEC and HPVe/pOVEC by activation of the fp2u TATA box segment of HPV-16 P97 through the SV40 or the HPV-16 enhancer. This transcript was strongly reduced in the fp2u* Sp1 mutant vectors (lanes 2 and 3, 5 and 6, 9 and 10, and 11 and 12, ct). Comparison of lanes 11 and 13 (cells were cotransfected with HPVe/pOVEC and pBLCAT2-E2) shows that P97 activity was only slightly modified in the presence of BPV-1 E2 protein expressed from pC59. A test vector with E2-binding sites in enhancer configuration was strongly stimulated, as visualized by band cat-t, which was visible in lane 11 only after long exposure. The RNA from a reference vector (ref) permitted monitoring of similar transfection efficiencies. The readthrough (rt) signal comes from an unidentified start upstream of P97. Lane 7, Sequencing lane for molecular weight determination.

upstream of the herpes simplex virus-thymidine kinase-CAT fusion gene (see Materials and Methods).

P97 activity is virtually completely dependent on a functioning Sp1-binding site (Fig. 6d, lanes 11 and 12, bands ct). In the presence of large BPV E2 protein (Fig. 6d, lane 13, band ct), P97 function is slightly increased. A dramatic increase of 1 or 2 orders of magnitude of thymidine kinase promoter function under the influence of E2 protein occurred when E2 binding occurred in enhancer configuration relative to the test promoter rather than overlapping with it (Fig. 6d, lanes 11 and 13, band cat-t). We conclude that under our test conditions in the presence of Sp1 binding and in the presence of two functional E2-binding sites, P97 activity was not strongly affected under concentrations of E2 protein which suffice to activate a test system for enhancer function of E2 protein.

DISCUSSION

Research into papillomavirus transcription is dominated by the analysis of the viral E2 transcription factors, a complex viral feedback regulation system of transcription enhancement and annihilation or even reversal of these stimulatory effects (for reviews, see references 31 and 39). In contrast, research into the interaction of the viral genome with cellular transcription factors has been somewhat neglected. Recent publications have documented, however, that important parts of the viral life cycle are encoded in *cis*-responsive elements recognized by cellular factors such as cell type-specific transcription (14, 22), stimulation of E6/E7 transcription and of transforming properties through certain steroid hormones (7, 22, 37), or mediation through AP1 sites by phorbolesters, second-messenger signals, and certain tumor genes (6, 21), and it is conceivable that this transcriptional induction is one step in genital carcinogenesis (4). Some further transcription factors that activate HPV transcription, such as NF1 (24), are recognized as being of qualitatively ubiquitous distribution in various cell types, but recent publications have revealed significant quantitative differences and qualitative heterogeneities (9, 27, 41) of some of these factors whose functional significance still has to be elucidated. Sp1 is another factor often considered to play a role in the unregulated activation of promoters and enhancers but has recently been shown to be influenced by glycosylations close to its transcription activation domain (28).

Our data showed that an Sp1-binding site is a distal element—possibly the only one recognized by cellular transcription factors—that is essential for the function of the major early transcription start, the E6/E7 promoter of genital HPVs. Two protein-DNA complexes formed on the corresponding elements of HPV-16 and HPV-6/11 that were indistinguishable in binding specificity and were recognized by a polyclonal antibody preparation from those complexes that form on the bona fide SV40 early promoter Sp1 sites. We do not have information as to the differences between the two complexes that form on Sp1-binding sites, although one could speculate that they represent different modifica-

tions of the same protein (28). The same complexes and a similar reaction with the same anti-Sp1 antiserum have been observed previously, for both an SV40 Sp1 oligonucleotide and a sequence representing a natural gamma globin gene promoter mutation (46).

Our experiments point to the danger of interpreting the presence or absence of certain sequence motifs as information about transcription factor-binding sites by showing that the classical GGGCGG motif of the Sp1 factor diverged among different HPV motifs and retained the G residues in position 2, 3, and 5 as the only common bases, a relaxed sequence requirement of Sp1 also reported for the promoter of the human growth hormone gene (33).

Future research will have to clarify whether the preference of genital HPVs for Sp1 distal elements fulfills some requirement, for example, either by supplying a defined transcriptional environment in the different layer of an epithelium or by permitting peculiar forms of cooperation specific for the particular composition of the enhancer of genital papillomaviruses as summarized recently (10). Speculatively, papillomaviruses might also have a tool such as SV40 to selectively enhance the concentration of Sp1 to create a more favorable environment for the function of their own promoter (40).

We have inspected sequences upstream of various papillomavirus promoters (2, 8, 30) either for the relaxed sequence requirement of Sp1 or for CCAAT boxes which bind NF1/CTF, a factor that has a strict requirement for TTGGC motifs (for discussions, see references 9 and 24). Besides the E6/E7 promoters of the genital papillomaviruses, BPV-1 P7940 has at position 7855 to 7866 both an Sp1 and an NF1/CTF site, HPV-8 (20) has at position 64 to 68 an NF1/CTF site, and HPV-33 has at position 9 to 13 an NF1/CTF site, whereas BPV-1 P7185, PL, P89, P2443, and P3080 and HPV-6b P270 do not exhibit either of these sequence elements; knowledge of their requirements for activating transcription factors has to await future research.

A line of our future research will address the question of what effect the E2 protein-binding site next to the Sp1-binding site has as to the simultaneous or competitive binding of these 2 factors. It has been shown that Sp1 displaces the transcription factor GHI in the promoter of the human growth hormone gene (33). A similar behavior might be expected for the papillomavirus promoters, whose Sp1- and E2-binding consensus elements are only spaced by 1 bp in contrast to the 7-bp spacing of the growth hormone promoter, unless steric properties of these proteins permit neutral or positive interactions.

A detailed study of whether Sp1, E2 proteins sites, and the components of the transcription initiation complex are binding P97 sequences in a competitive, cooperative, or noninterfering manner will be a prerequisite for understanding the fine-modulatory regulation governing the E6/E7 promoter. Mutational studies have shown that the promoter-proximal E2-binding site leads to promoter down regulation in the presence of E2 protein (39, 48). The discrepancies between these data and our findings reported here could be resolved if E2 protein bound to the distal element would give a positive effect and if E2 protein bound to the proximal element would give a negative effect. The observed outcome of an experiment may depend on the fraction of each site being occupied in the population of test vectors, which depends on variables such as the transfection protocol.

It has been proposed that absence of E2 protein, e.g., after insertional activation of the E2 gene, may be an important step in cervical carcinogenesis (43), a view challenged by the identification of tumors with (most likely) intact E2 open reading frames and even episomally replicating HPV DNA (11, 19, 50). As a modification of this view, our data suggest that E2 protein may be expressed without reaching the concentration necessary for a down-modulatory effect. E2 protein responsive elements as well as binding sites for cellular transcription factors in HPV enhancers-promoters are likely to function as tools to fine-regulate HPV transcription according to the viral life cycle or to physiological changes encountered by the host cell.

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